

21 January 1982

Can change damage your mental health?

The US Court of Appeals says that environmental impact statements should cover psychological factors, giving environmental pressure groups a licence for mayhem.

The United States Appeals Court in Washington has put a pistol, perhaps even a bazooka, into the hands of opponents of new developments of any kind. The court has now ruled (see page 179) that electricity cannot be generated at the undamaged reactor at the Three Mile Island site on the Susquehanna River in Pennsylvania until its luckless owner has shown that the mental health of the local population will not as a result be damaged. The decision is important not merely because it may be the straw that breaks the back of Metropolitan Edison but because it can in principle apply to any project caught by the Natural Environmental Policy Act, 1970.

So far, impact statements have been attempts to anticipate physical, biological and social consequences of new developments. In many ways, they have been interesting and even important documents. The impact statement on the Alaskan pipeline may even now be the handiest source of information on the migration of caribou, for example.

Impact statements as they are suffer from a common imbalance. The most tangible and easily anticipated consequences of some new development are covered in great detail. The species of flora and fauna within striking distance of a development site are usually catalogued in detail, as are the volumes of traffic expected on the highway system. (Farsighted developers do their best to recruit the help of the local university in carrying out this kind of work.) The less tangible but usually more important issues raised by new development projects are, perhaps inevitably, given less close attention. While it may be possible to estimate the long-term consequences of a new development for the economic development of a neighbourhood, even that kind of calculation is necessarily shot through with sources of error. And so far, those responsible for preparing impact statements — itself a kind of growth industry — have shrunk from trying to estimate whether a development will make the local population happier. The importance of last week's decision is that it requires not merely the owners of the Three Mile Island nuclear site, but in principle all other managers of public works in the United States, to show that their proposals will not bring psychological damage.

The objective is laudable but the task which it implies is both impossible and, on grounds of principle, irrelevant. What has happened around Three Mile Island since the accident in March 1979 should have given the Appeals Court pause. The population in the neighbourhood of the reactor has understandably been a focus of interest for social scientists seeking evidence of the minor psychological consequences of such an occurrence. Most accounts of these investigations appear to agree that during the immediate crisis, people living within fifteen miles or so of the damaged reactor consumed more sleeping pills than those living further off. Some of these habits persisted for as much as nine months, but Dr Peter Houts of Pennsylvania State University, the author of one of the most thorough investigations of this kind, says that his latest survey, in October last year, shows no residual measurable consequences of the reactor accident. Other studies, reported by the Kemeny Commission, suggest that at no stage were the signs of stress reported by the local population sufficient in frequency and intensity to qualify as psychiatric illness.

Against this background, it is inconceivable that environmental impact statements purporting to describe the psychological impact of some new project can be more than fanciful fiction. It is

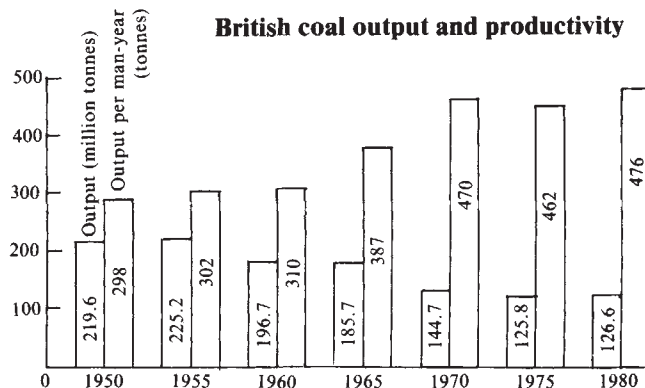
just conceivable that, as at Three Mile Island, it might be possible to measure something tangible. But how, in the absence of a range of studies in the neighbourhood of sites capable of generating serious accidents will it be possible to predict the psychological stress caused by some new development? Will the federal government be expected now to sponsor essential baseline investigations of the incidence of minor symptoms of anxiety among people living near, say, Titan missile silos, or projected missile sites as well as nuclear reactors? What is the chance that the results will be statistically significant? And what use will they be to the drafters of future environmental impact statements?

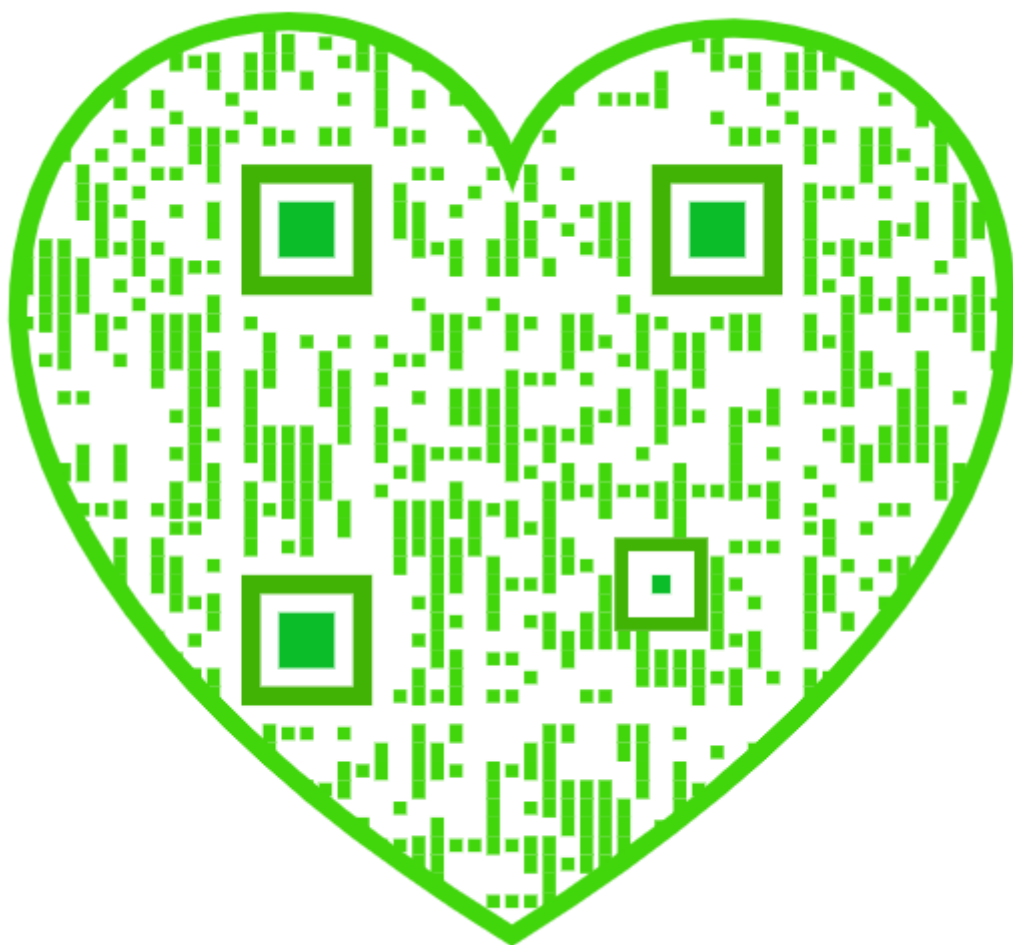
Even if the measurements the court supposes could be made were made, however, they would be irrelevant. Provided that development projects are not so awesome that they topple people over into frank psychiatric illness, the all-important question becomes that of whether there should be a moratorium on all development projects likely to increase the general level of anxiety in the population. Presumably hydroelectric schemes of all kinds would quickly be stopped, however necessary their output of energy. So too would most defence projects and all nuclear projects. The result would be a nonsense, so that the court's decision must be changed. But that will take time. Meanwhile, environmentalists have a licence to do irreparable damage.

Paying the price for coal

The British coal industry, again in crisis, should be either closed or administered logically.

In February 1974, the then Conservative government called a general election in the wake of a damaging strike about pay by British coal miners. The central issue at the election was the government's question "Who governs Britain?" — the elected government or the miners. The rhetorical question was never answered, although Mr Edward Heath, prime minister at the time, was clearly told that it should not thereafter be him. A similar tangle with workers in nationalized industries among whom the coal miners were again conspicuous put paid to the government which succeeded him, in the spring of 1979, and paved the way for the present administration. It is therefore inevitable that Mrs Thatcher and her colleagues should be nervous about the outcome this week of the ballot among the miners, who are being





asked by their leaders to agree that there should be another strike, again over pay, if one should "be necessary". Pollsters and soothsayers guess that, this time, the voting will be so close that there will not be a clear mandate for a strike, in which case the government will claim a victory for good sense and for its policies of the past two years, miners' pay will increase by 9.3 per cent and everybody will forget about the issue until next year. That would be a serious mistake. What the British government should now be asking is whether Britain needs a coal-mining industry, or can afford one.

On strictly economic grounds, the mining of coal in Britain should be stopped. The cost of producing and distributing British coal within the United Kingdom is more than that of importing it from the United States and even from Australia. For several years, the nationalized National Coal Board's chief customers, the nationalized Central Electricity Generating Board and British Steel Corporation, have been muttering about the advantages of importing coal, and did indeed buy 5 million tons of coal from overseas in 1980–81. Given encouragement, these two customers for domestic coal production (which take well over three-quarters of total output) could profitably import more. In reality, they are being pushed in the other direction by the National Coal Board and by the miners, chiefly by being offered British coal at prices well below the average cost of production. The British government provides a modest subsidy to this end, but not nearly enough to make good what the coal board loses on the transactions. Two consequences follow inexorably. By hanging on to its chief domestic customers, the coal board is forced to charge others more than would strictly be necessary. But, even so, the coal board's accounts are perennially in deficit. The British government, although perpetually preaching economic realism, and the coal board are in collusion to make water run uphill.

Although this curious state of affairs has arisen because of the determination of British coal miners not to let production fall still further — it has halved in a quarter of a century — it should have been no surprise. More than a century has passed since W. Stanley Jevons argued in *The Question of Coal* that British industry as a whole was vulnerable to competition from abroad because of its dependence on deep-mined coal, inherently more costly than the coal from thick near-surface deposits such as those in the plains states of North America. (Forgivably, Jevons did not anticipate the phenomenon of Japan, industrially competitive with hardly any indigenous fossil fuel.) The British coal industry, like the more heavily subsidized coal industries of Belgium, France and West Germany, is victim to the geology of all extractive industries. The British coal board would be more competitive if it were more free to concentrate production on those coal fields in which seams are thicker and more accessible, but its employees will not let that happen. The board's plan to shut twenty uneconomic coal mines during 1981 was modified after a series of strikes, and in the event only ten were shut. Given that failure, economic logic would suggest that British users of coal should be encouraged to meet their needs from abroad, and that the British coal-mining industry should find its own presumably shrunken level at which costs and prices are in some kind of balance.

There is no prospect of this happening. For half a century, the politics of coal mining have overridden economics and common sense. In present circumstances, the coal board has a minor point in its arguments that the cost of imported coal is now lower than it would be in normal times because the cost of shipping is depressed by the world recession. The board and the government are, however, united in their belief that the British coal-mining industry is strategically necessary — a hedge against the emergence of an Organization of Coal-Exporting Countries or some other improbable development of that kind. The argument would be more convincing if it were made explicit, and if its implications were followed consistently. First, there is no reason why strategic considerations should require 100 per cent self-sufficiency. Why not 80 per cent, or 70 per cent, or some other proportion of present consumption? Second, if the high cost of domestically produced coal is politically and financially an embarrassment, the British government should become the active

sponsor it once promised to be of the only other substantial source of indigenous energy — nuclear power stations. Third, and most immediate, if the case for keeping alive the British coal industry is strategic, should the government continue to insist that the substantial hidden costs of that policy should fall primarily on those who use it? The result is that the coal board has to sell its product at too high a price to unwilling customers, thus ironically undermining the objectives of keeping some kind of coal-mining industry in good shape — and needlessly increasing industrial costs at the same time. The British government, which has enthusiastically embraced its predecessor's policy of charging the international price for oil from the North Sea, should now do the same for coal. Then at least the cost of the present policy would be explicit in the government's accounts and not hidden in those of the coal board.

Ice age or melt-down?

Recent winter mid-latitude weather has been awful. But it is a fluctuation, not a cataclysm.

After a couple of weeks of unexpected but seasonal cold weather in Western Europe and North America, the public rumour mills are grinding out news that there may be another glaciation around the corner. A host of people who should know better, many of them professional earth scientists, have been engaged in grave televised discussions about the chances that a few days of snow and slush either presage the return of the ice sheets (this time without bison and mammoth) or are paradoxical accompaniments of the general increase of atmospheric temperature to be expected from the accumulation of carbon dioxide in the atmosphere. Television interviewers have been visibly discomfited by this apparent assault on common sense, and have usually chosen to leave their audiences with a reminder that the ways of science are incomprehensible, and that if a suitable qualified gentleman has said that it has been devilishly cold because the earth is getting warmer, he must be right.

The truth, of course, is that none of this rambling is relevant to the past weeks' weather, in Western Europe, North America or anywhere else. The meteorology of Western Europe's admittedly wretched weather is easily described and is even familiar. The usual stream of depressions across the Atlantic was diverted from course by an apparently stationary centre of high pressure over Scandinavia. As a result, relatively warm southwesterlies were replaced by cold northeasterly winds. Closely similar stationary high-pressure centres occur most winters inland of the western seaboard of Eurasia, but not always at the same longitude. The intriguing question is why such high-pressure centres should be stationary. The simple answer is that nobody knows. The more complicated answer is that many people are trying to find out. Whether the frequency or durability of such high-pressure centres are positively correlated with the atmospheric concentration of carbon dioxide is plainly an unanswerable question, and will remain so until the mechanism of what the meteorologists call blocking is understood.

So is there no choice but to regard the past few weeks' weather as a statistical fluctuation on the usual pattern? None. Are the climatic consequences of all that extra carbon dioxide still in doubt? Yes. And is there a prospect of being able to walk once more from Britain to France as at the peak of the most recent glaciation 18,000 years ago? No, too little is known. That, when the next season of blizzards (or of droughts) comes round, is the public message that the professionals should seek to put into circulation. It is something of a triumph, for the public understanding of the world we occupy, that people's imaginations have been caught by knowing that there is no way of distinguishing between the present period and one of the earlier Pleistocene interglacials. It serves no purpose to corrupt that state of affairs with over-sharp predictions of what the future holds. One swallow does not make a summer, nor one blizzard a glaciation.

Mental stress given environmental status

Court delays start at Three Mile Island

Washington

In a precedent-setting decision, the US Court of Appeals in Washington has decided not to allow the Metropolitan Edison Company of Pennsylvania to switch on the undamaged power plant at Three Mile Island, near Harrisburg, until it can demonstrate that such a move would not adversely affect the mental health of people living in the area.

The immediate impact of the decision, which reverses a ruling by the Nuclear Regulatory Commission (NRC) that psychological factors do not necessarily have to be taken into account in deciding whether to allow nuclear plants to start operating, will be to delay for several more months the start up of "Unit One" at Three Mile Island. The reactor was undamaged in the accident which occurred to its twin, Unit Two, in March 1979, and since then has been considerably modified to improve safety.

The ruling, however, which was made by two of the three members of the appeals court, could have a significantly wider impact. For the first time it means that although the powerful Natural Environmental Policy Act of 1970 does not require psychological factors to be taken into account when the social impact of a federal decision is being assessed, the act can be interpreted as allowing such factors to be included.

The Three Mile Island accident resulted in a small amount of radioactivity being discharged into the environment but no significant extra radiation exposure to individuals, so nobody suffered physical health damage as a result. The principal focus of interest, therefore, has been on the psychological implications of the accident.

Arguments about the psychological impact of the accident have been continuous since it occurred. Civic leaders warned NRC that thousands of people might be driven from their homes by fear of radiation if krypton gas was allowed to be vented from the crippled plant. Some claimed that "riots" might result as a possible manifestation of the population's nervousness.

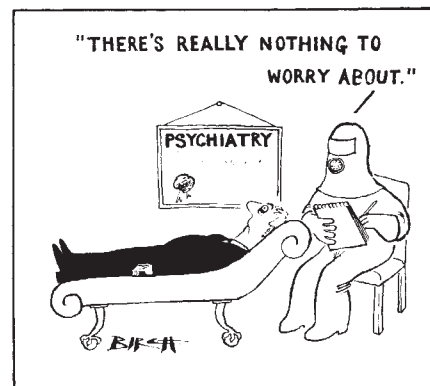
Such arguments have, until now, fitted uneasily into the institutional mechanisms set up to cope with the social consequences of major technological actions. Thus when Metropolitan Edison applied to NRC to be allowed to start up the undamaged plant, the commission rejected claims from a local citizens' group, the People Against

Nuclear Energy (PANE) of Middletown, Pennsylvania, that such a move would have sufficient psychological effects to make it necessary to take these into account.

The request from PANE had been supported by the members of NRC's own Atomic Safety and Licensing Board — partly on the grounds that taking such factors into account while the licensing decision was being reached could head off later conflict — but was rejected by NRC.

The Appeals Court has now told NRC that it was wrong, and has directed the commissioners to conduct a broad assessment of the effects that starting up Three Mile Island's Unit One will have on "the psychological health of neighbouring residents and on the well-being of surrounding communities". After that, the commission will have to decide whether the National Environmental Policy Act requires a more detailed analysis of the social and environmental impact — possibly including a public hearing — before the decision to restart the undamaged reactor could be taken.

The court's decision was not unanimous. In a strongly-worded dissent, Judge Malcolm Wilkey argued that by establishing psychological stress as a factor which had to be taken into account by NRC, the court was defining "an 'impact' which has never before been considered as covered by the National Environmental



Policy Act", a move which, he said, had "enormous consequences".

The decision was greeted with gloom at Metropolitan Edison, which had been hoping to start up Unit One within the next few days, and which was depending on revenues the reactor would produce from the sale of electricity to help cover some of the costs of the clean-up at Unit Two which are already threatening to force the utility into bankruptcy.

Predictably, the appeal court's decision was welcomed by members of PANE. The group had argued that the inclusion of psychological factors should be part of the responsibility of licensing institutions to protect the public health and safety, as defined in the Atomic Energy Act of 1954.

David Dickson

European nuclear safety policy attacked

Brussels

Has the Euratom treaty, which binds the European Community's nuclear energy policies together, been properly interpreted and applied? A lengthy report by the leading Belgian socialist member of the European Parliament, Anne-Marie Lizin, suggests not and attacks the lack of progress towards achieving the aim of a European zone of unified nuclear safety standards.

Lizin's report was the subject of heated debate in the European Parliament's Committee on Research and Energy, where 91 amendments were proposed. Having looked in detail at EEC's activities in the fields of radiation protection, reactor safety, decommissioning, safety of the fuel cycle and the national nuclear inspection services, Lizin concluded that the Euratom treaty is outdated and vague and that the European Commission has made too little use of its powers under the treaty.

Lizin alleges that when the treaty was first drawn up, more than 20 years ago, too little consideration was given in the nuclear energy development plans to reactor safety and worker protection. Since then, the member states have proved reluctant to provide the European Commission with effective consultation procedures for all

decisions concerning the location and operation of nuclear power stations. In Lizin's native country, Belgium, the licence to build a nuclear power station is granted independently of the initial safety analysis, and given the fact that too many nuclear power stations are built close to frontiers or use shared river systems, the report complains that there is too little consultation on measures to protect workers and local populations.

The report goes on to say that although the Community has been efficient at listing methodologies, standards and safety criteria, work on listing similarities and differences among the member states has been too slow and the Community has yet to go on to the third stage of putting forward its own regulations. This work has largely been left to other international bodies such as the Organization for Economic Cooperation and Development and the International Atomic Energy Agency. Lizin attributes particular importance to the Super-Sara project but calls for more studies into the safety of gas-cooled and heavy water reactors. The problems of waste management and recycling are given particular attention because the report considers that this field requires intense supervision by an independent public

authority.

The role of the Community in the decommissioning of nuclear plants is reassessed. Lizin suggests that EEC's financial instruments, such as the regional fund, could help and that electricity companies or utilities should be asked to pass on decommissioning costs directly to the consumer. Lizin's lengthy list of recommendations also tries to sting the Commission into providing stricter surveillance of the work carried out by national supervisory authorities.

Undoubtedly something akin to the steel crisis, which made many realize the immense emergency powers invested in the Commission by the treaty of the European Steel and Coal Community, will be necessary to bring the strength of the Euratom treaty into play. But Lizin's awareness of this possibility has been heightened by the controversy surrounding the enlargement of the French nuclear power site at Chooz, a few kilometres from the border with Belgium. Notwithstanding local and national protests from Belgians, and the French socialist party's preelection promises, the French Prime Minister, François Mitterrand, still seems set on increasing the number of nuclear reactors there.

Jasper Becker

French colloquium

Science passion

Paris

The great national colloquium last week on the state of science in France was sometimes like a high mass, sometimes more like a giant committee meeting. And although, after six months preparation, the colloquium (which was strictly advisory) displayed few radical disagreements, the test of its success will come only with the publication of a new law to be put to the French Parliament in the next few weeks.

The theme running through the four-day meeting was uplifting. M. Jean-Pierre Chevènement, Minister of State for Science and Technology, told the closing session that "the highest authorities of the state" have decided that scientific research is vital "for the successful change and future of our country". The highest authority himself, President François Mitterrand, had set the ball rolling with a declaration that "only a gigantic effort in research" will allow France to master its technology and thus make certain of its independence.

Both the President and his Prime Minister, M. Pierre Mauroy, were, however, anxious that the forthcoming expansion of science in France should follow the "wishes of society". Mauroy also wishes to reintegrate science into the wider culture of France from which he thinks it has been too long isolated. But to Chevènement himself, "science is a passion and France needs passion". Since the election in May 1981, he said, hostility

River of words, mountain of paper

Paris

There were no *bateaux mouches*, the "firefly boats" with glass roofs, on the Seine last week. The river was a torrent almost filling the arches of the bridges and menacing the city. It was much the same with *la vague Chevènement* (the Chevènement wave) as one paper dubbed it. The Minister of State for Science and Technology, Jean-Pierre Chevènement had organized all the tributaries of science in France, and in its overseas territories and departments, to converge on Paris.

The organizers of this national colloquium for science and technology estimated that there had been nearly 200,000 pages of written contributions to the colloquium and its regional predecessors (held in October and November last year). This is almost a page for each of the 280,000–300,000 scientists and technologists in France. There were almost 10,000 individual documents, all of them arguing over which points needed to be changed, modified or amplified in the politics and organization of science and technology.

A torrent of words, a torrent of ideas and clearly an historic moment for French science: Chevènement certainly hopes so, for his ambitions go beyond science and technology. Last week, he did his best to take strict control of the colloquium. The 200,000 pages were reduced to 950 reviews, written largely by people chosen by the ministry. Then 200 of these reviews were placed before 12 colloquium committees, also hand-

picked. And the committees reduced these to 40-page summaries, which were then discussed.

Neither colloquium nor committees were empowered to take decisions. And even the most apparently practical



committee, that on finance, was required to discuss only principles: such sordid matters as actual figures and percentages were set aside.

Nevertheless, there had been attempts to make the composition of the committees representative. There were more than 100 people in each committee, making 1,500 in all, drawn from three categories — 200 were directly involved in preparing the national colloquium and the regional summaries, 500 were from institutions, such as industries, the principal science funding bodies, ministries, universities and unions.

Robert Walgate

to science and technology had been turned back.

Inevitably, on what could not fail to be a patriotic occasion, the minister also developed his theme that French science must increasingly be seen to be French. He promised data banks and scientific books in French and that the great international science journals would be translated. Meanwhile, the French cabinet has agreed that the volume of government spending on science should increase by 17.8 per cent a year over the next three years and that private industry should be encouraged to increase research and development investment by 8 per cent a year. This should bring French research and development expenditure to 2.5 per cent of the Gross National Product by 1985. Jobs will be created at the rate of 4.5 per cent a year over the same period.

All this has given the minister for science more spending power and more influence. He already controls almost all the forward-looking agencies of the Ministry of Industry and is thought to be lobbying for the merger of that ministry with his own.

Chevènement is nothing if not visionary and ambitious. The politics of health

depend on research, he said, as do the politics of labour and employment. Science and technology could increase French independence while the creation of a "French pattern" for the organization of science could be a model in fields other than science and technology.

The new law, to be prepared in the next few weeks, will define new contracts of employment for researchers, set financial targets for institutions and set the "French pattern" of research for 1983–85. The expectations are that the law will leave most institutions unchanged except for the introduction of union representatives on certain committees. But the law will provide for new small-scale technical enterprises and regional centres of development in certain fields.

Under the promised arrangements, there is to a new high-level advisory council together with regional advisory committees. Social science, Chevènement said, needs to be impelled forward. And what is it all for? "To spread knowledge, to push back anti-scientific prejudice, to assure the progress of the sciences of man and society, to maintain or re-establish French."

Robert Walgate

University of London

All change now

Of the central functions of the University of London, only the students' union is wholeheartedly applauded for its efficiency in the final report of the Committee on Academic Organization, published last week. The committee, set up in March 1980, was originally intended to recommend a new organization for the university. After last summer's financial cuts, the committee's terms of reference were redrafted to exclude the main teaching institutions.

One conspicuous recommendation in the report is that the university's Nuclear Reactor Centre (at Silwood Park in Berkshire) should either be closed or, preferably, transferred to Imperial College. The committee says that the centre's chief function is research, and that it should be possible for the centre to find at least half its total cost of £220,000 a year in research grants.

Among other recommendations are the continuation of the Marine Biological Station on the Clyde (administered jointly with the University of Glasgow) and the Institute of Archaeology. The report also suggests that the university could save £100,000 a year by closing its Botanical Supply Unit.

The most serious criticisms are of the central administrative machinery. The court department (which shares out the university's public grant) is too secretive, and urgently in need of computer techniques, the examinations department is overstaffed and the university's network of academic committees has outlived the time when "the need and even the opportunity for taking genuine decisions . . . has ebbed away".

In general, the committee comes down in favour of a university in which its colleges function autonomously, taking more control from the centre. But it is also against the proposal that University College should be financed separately.

These proposals are for change within the existing framework. The committees set up last September to recommend new patterns of teaching in broad subject areas appear to be following an orthogonal track. The first of their reports, published this week and dealing with the physical sciences, starts from the assumption that only large departments can be successful in research.

Most of its recommendations are that the teaching of science subjects should continue at four colleges (Imperial, King's, Queen Mary and University) and at most one other site within the university. A total of 58 academic posts in the physical sciences would be lost.

Both sets of recommendations will excite opposition. In each case, the next step is discussion by the university's academic committees and the senate.

AT&T pact hits snag

Washington

A federal judge in Washington has injected an element of uncertainty into plans to reorganize the giant American Telephone and Telegraph Company (AT&T) by announcing that, although the federal government and the company have agreed on terms for suspending the anti-trust suit filed by the Department of Justice, he is unwilling to dismiss the suit without further study of the agreement.

The Reagan Administration announced the settlement with AT&T, the largest company in the world, on 8 January. Under the terms of the agreement, AT&T has agreed to sell off 22 local telephone companies, with assets worth around \$80,000 million, in return for being allowed to enter other fields of telecommunications, such as electronic data processing, from which it had previously been excluded.

US District Court Judge Harold H. Greene, who has been hearing evidence in the government's case against the company since 1978, said last Tuesday that he was "delighted that a settlement had been reached". At the same time, however, apparently concerned at the secrecy and speed of the negotiations between the company and the Justice Department, he has refused to dismiss the case without further scrutiny of the terms of the agreement. Judge Greene said that the case was "too important to the judicial process, to the public and to the country" to be closed as a result of a "haphazard process".

Judge Greene's actions are in line with a law passed by Congress in 1974 giving the public an opportunity to comment on anti-trust agreements between the government and a private company before these are concluded. The law was passed in partial protest at the secrecy which had surrounded negotiations between the government and AT&T leading to a previous consent agreement in 1956.

David Dickson

Polish universities**Wings clipped**

Polish universities will resume work by mid-February, according to a communiqué issued on 10 January by the ruling Military Council of National Self-Defence. Tertiary education establishments, it says, will function "taking into account regulations deriving from the decrees on the state of martial law" and special rules have been laid down governing the running of full-time courses during the state of emergency. Moreover, resumption of university activities each term will be approved on the basis of recommendations from the Provincial

Defence Committee — on the basis of how, in the previous term, the university complied with the emergency regulations.

The communiqué effectively abrogates the liberalization of academic life which followed the signing of the Gdansk accords in August 1980. In particular: academic and scholarly work is to be censored. The abolition of the censorship of learned publications was one of the first demands of the Academy of Sciences and the universities in autumn 1980, and was embodied in the censorship law which came into force on 1 October 1981.

University autonomy concerning syllabuses will be restricted. All students will have to attend courses in two foreign languages. This means a return to compulsory Russian, which was abolished by the Lodz accords that ended the students' sit-in last February. New courses and subjects will require ministerial approval, which will presumably not be forthcoming for courses reflecting the "plurality of outlook" promised at Lodz.

University rectors will now be responsible for maintaining order on campus and in students' hostels. No student may remain on campus outside tuition and library hours, and all staff must leave as soon as their working day is over. This reverses the spirit of the Lodz accords which left law and order on university premises firmly to the university authorities and banned civil police from campuses.

Attendance at lectures and classes will be obligatory, and absence without sufficient excuse may result in expulsion. Students may also be expelled for infringing Article 46 of the decree of martial law, which bans union activity and the organization of strikes and protests. Special classes are to be held at the start of the new session, explaining to the students the demands of martial law, and particularly of the new oath of loyalty.

University senates and faculty councils will be reduced to advisory status, and will have a composition "determined by the regulations of the law in force". This provision is ambiguous, since the communiqué also says that the universities will operate on the basis of the 1958 law on tertiary education — which would mean an end to the 30 per cent student participation in senates and faculty councils introduced after Lodz.

The rectors are instructed to appoint special plenipotentiaries to deal with the welfare problems formerly the province of students' unions and self-governance bodies, and will have the right to order their employees to carry out tasks hitherto not considered part of their duties. They may also order students to carry out "socially useful work" for the university or the national economy.

During the past year, all Polish university and technical college rectors have been democratically elected — except in Radom Engineering College, where an attempt to impose a rector who was not so

Gentle Polish protest

The Royal Society and the British Academy last week sent a message of "sympathy and support" to the Polish Academy of Sciences. The message, signed by the presidents of the two academies, regrets "the interruption of normal relationships" between Britain and Poland and hopes "that they will soon be resumed". The message is to be sent to other academies which are members of the International Academic Union and the International Council of Scientific Unions.

elected led to nationwide student protests and a virtual shut-down of academic life. At least three rectors have been reported to have been interned: Dr Henryk Samsonowicz of Warsaw, Dr Jozef Gierowski of the Jagiellonian University of Krakow and chairman of the unofficial rectors' conference and Dr Janusz Ziolkowski of Poznan. How far other rectors will comply with regulations which seem aimed at reducing them simply to disciplinarians is unknown. **Vera Rich**

Finnish-Soviet research

Arctic collaboration

A new Finnish-Soviet Arctic research committee will meet for the first time next month, to work out a new joint programme for the development of the Soviet Arctic. The formation of the committee comes at a critical point in Finnish-Soviet relations: the retirement from politics of President Urho Kekkonen, the chief architect of Finland's policy of neutrality coupled with close economic cooperation with the Soviets.

Earlier this month, Mr Ahti Karjalainen, head of the Bank of Finland and chairman of the Finnish-Soviet economic committee, was in Moscow for talks on future deals including an icebreaker to be built for the Soviets in a Finnish shipyard and a contract, worth up to £125 million, for the third stage of the paper mills and urban development being built by Finnish expertise at Svetogorsk, just inside the Soviet frontier.

The new Arctic research committee, though essentially a research and development body, will to a certain extent overlap the economic agreements. For example, Mr Karjalainen has been discussing a joint off-shore geological survey for oil and gas in the Barents Sea. Accordingly, one of the major joint research projects on the agenda of the new committee will be devising the technology necessary to construct an oil and gas drilling rig on an ice floe.

Finland has considerable expertise to offer its partner. One participant in the new committee is the University of Oulu, which under the recent policy of decentralized and regionalized higher education runs a programme of research into the economic

and development problems of northern Finland, much of which can be immediately applied to work in Arctic Siberia. In particular, Oulu will contribute a new technique for surveying terrain with up to 2 metres of snow cover. Oulu is also the seat of the Research Council for Arctic Medicine, and has a special laboratory for the study of the physiological hazards of work in a cold climate.

The main workload of the cooperation will be undertaken by the Technical Research Centre (Valtion Tekninen Tutkimuskeskus, VTT), a government-sponsored body whose mandate includes research (at cost price) on behalf of those who wish to exploit the results but who do not have the facilities to carry out basic investigations themselves. VTT has 31 laboratories (26 in the Helsinki area, 3 in Tampere and 2 in Oulu). Fifteen of these laboratories will be involved in the cooperation programme, and it is estimated that participation will allow VTT to double its research staff in the next few years. Finland, with a population of 4.7 million, has no less than 18 universities, an escalation from two over the past 25 years resulting from "regionalization" policies, and employment for graduates who wish to remain within the Finnish academic/research structure is a perennial problem. A strong swing of public opinion away from science in the mid-1970s was by no means fully reversed by the then President Kekkonen's call for a reassessment of research and development investments in his New Year message to the nation in 1979. (This was the first time that science had been officially boosted since 1964.)

The new Soviet-Finnish Arctic research programme, the details of which will be worked out next month, is already being regarded by Finnish economists and business experts as a major shot in the arm. Its effect on graduate employment should prove equally beneficial. **Vera Rich**

Deep-sea mining

United States re-think

Washington

United States policy on deep-sea mining is again in the melting pot. The recent discovery of large ocean-bed deposits of polymetallic sulphides in various parts of the world has significantly raised the stakes in the continuing disputes over what form of international regulation should be introduced to deep-sea mining.

In the United States, plans are already being discussed to amend the Deep Sea Mining Act, passed by Congress only two years ago. The act provides a mechanism for issuing licences to US companies wishing to collect manganese nodules from the sea bed but could, as it now stands, exclude retrieval of the sulphides.

A different problem exists with the UN Law of the Sea Treaty, negotiations on which are nearing completion after eight years. The current draft of the law is sufficiently flexible

to include polymetallic sulphides but US mining companies, backed by the State Department, are concerned that in its present form with no explicit attention to the sulphides, the whole question could be put on hold indefinitely.

Although chemical geologists have long predicted the existence of sulphide deposits associated with rift phenomena on the ocean bed, the first discovery was made as late as 1978 on the sea floor of the East Pacific Rise, near the coast of Mexico. Since then, several similar sites have been discovered off the Pacific coast of the United States, for example in the Juan de Fuca ridge off the state of Oregon. The minerals involved include cobalt, manganese, platinum, zinc and tin.

The most dramatic discovery so far, however, was announced last October by scientists from the National Oceanographic and Atmospheric Administration (NOAA) of the US Department of Commerce. The NOAA geologists have found several mineralized zones between the Galapagos Islands and Ecuador, in the Pacific, which they estimate to contain 25 million tons of polymetallic sulphides. The high concentration of copper and iron in the sulphides is calculated at about 10 per cent each. Also present are lead, molybdenum, vanadium and zinc (0.1 per cent each), and silver and tin (0.3 per cent), with trace amounts of cadmium, gold and platinum.

Whether the minerals in the sulphide deposits are sufficiently accessible to be worth mining is yet to be determined. The NOAA geologists estimate that the total value of the metals in the latest find is about \$3,000 million, and new mining and processing techniques would have to be developed to retrieve them.

Several large companies such as the consortium headed by Lockheed Missiles and Space, are already turning their attention to the commercial potential of the sulphides and are carrying out preliminary exploratory surveys.

Whatever is discovered, mining of the sulphides will inevitably be affected by the negotiations that have taken place within the United Nations since the resources of the high seas were declared, by an unanimous resolution passed in 1970, to form part of the "common heritage of mankind", whose exploitation should be regulated in a way that would result in the most equitable distribution of benefits.

Deposits relatively close to national coast lines are unlikely to be covered by international legislation. In line with provisions contained in the draft Law of the Sea, the US Congress is expected soon to begin debating a bill which would create a 200-mile exclusive economic zone off the US coast. The discovery of the polymetallic sulphides will add to the political attractions of this bill, since several of the known deposits — such as those in the Juan de Fuca ridge — lie wholly or partially within this distance of the shore. And if the bill passes, as seems likely given the expected support of the Administration, the companies could begin mining the

deposits free of international regulation.

The deeper deposits, such as those off the Galapagos Islands, will create more of a problem. The deep sea mining bill passed by Congress two years ago was designed to provide US mining companies with certain rights that would have to be recognized in so-called "grandfather clauses" in any international treaty, since the latter would require ratification by the US Senate.

This law would require amendment in several places. For example, "hard mineral resources" are defined as any deposit or accretion of "nodules" lying "on, or just below, the surface of the deep sea-bed". Similarly the deep sea-bed is defined as including the subsoil "to a depth of 10 metres", while polymetallic sulphide mineral deposits may extend considerably further.

State Department officials are confident that, with the amendment of certain key definitional provisions, the act can be extended to cover the sulphides. There is much less confidence about the implications of the Law of the Sea negotiations, which Mr Otho Eskin of the department's Office of Oceans Law and Policy says raises "a very serious question".

Accepting that fundamental changes are unlikely to be agreed by the negotiators when they meet again in New York in March, and that a treaty in some form is likely to be adopted by the end of the year, the United States has two main alternatives. Either it could sign the treaty (which would be taken as a symbolic victory by the developing countries, for whom the treaty represents the first concrete manifestation of the "New International Economic Order"); or it could refuse to sign (which could jeopardize the chances of United States-based mining consortia obtaining the necessary loans from the multinational banks).

David Dickson

Indian agriculture

Cattle go West

Bombay

India's ambitious programme of cross-breeding its cows with foreign bulls is endangering the country's agriculture by creating a shortage of draught animal power — the mainstay of farming and rural transport in India. This is the finding of a survey covering the cross-breeding programme in 18 of the 22 Indian states carried out by the Bombay-based All-India Agriculture and Cow Protection Society (AIACPS) founded by Mahatma Gandhi 40 years ago.

A five-member team of veterinarians headed by Dr M. Y. Mangrulkar contacted over 1,600 cattle breeders and government crossbreeding farms for the survey. Their report just published says that the craze for "westernization" of Indian cows has resulted in the neglect of indigenous milch strains of cattle and has

led to shortage of good, sturdy bulls for farming operations.

The survey has revealed that exotic crossbreeding has mostly produced bullocks which are uneconomical. The exotic animals need special grade grass and oil cakefeeds and are unsuitable for the rigours of Indian agriculture: they get easily tired and are unable to walk in the sun or plough in wet fields, unlike the docile native bulls which live on paddy straw and are at ease in dry and irrigated fields. More importantly, the exotic crossbred males have no hump and the traditional ploughs do not fit on them. Thus the crossbred bullocks have been left to starve and die or been sent straight to the slaughter house. The AIACPS report says the crossbreeding programme, mostly financed by international agencies, is actually preparing India to become the world's leading exporter of beef to the meat hungry West. The slaughter of male calves is leading to a shortage of bullocks particularly in Bihar state where, the report says, thefts of bullocks are increasing.

In states such as Karnataka, crossbreeding has been in vogue since the 1950s but other states have taken to crossbreeding in the past two decades and the



programme was vastly expanded in the early 1970s under the World Bank financed "Operation (milk) Flood". According to the survey, the high pitched crossbreeding programme is weaning the countryside away from indigenous cattle in favour of Holstein-Friesians, Jerseys and Red Danes, and good Indian breeds are being replaced with unproductive crossbreeds.

The programme has led to the import of bulls, semen, vaccines and insemination equipment but has failed to increase milk production. The hybrid cows after second calving yield less milk than the high-yielding native breeds such as Kankrej, Sahiwal, Gir and Haryana. The survey also revealed that exotic crossbreeds are more susceptible to disease than native stocks, which has led to increased import of vaccines.

However, government officials maintain that the survey findings are at variance with their own evaluation. According to Dr R.M. Acharya, deputy director general of the Indian Council of Agricultural Research, Indian cows are not being westernized wholesale. "Only five per cent of the cattle population is exotic", he says and, the humpless crossbred bullocks are gradually becoming adapted to Indian

conditions and their number in farming operations is steadily increasing. According to Dr Acharya the crossbreeding is not new to India where all the military farms have been using exotic breeds for several decades. While India has indeed some good breeds, according to Dr Acharya, only a small per cent of them are really high milk yielders. Despite the controversy officials maintain there is no alternative to exotic crossbreeding for increasing India's milk production, which is currently estimated to be 23 million tonnes annually.

K.S. Jayaraman

Bridging the Indian gap

New Delhi

Mrs Indira Gandhi, the Prime Minister, announced last week a substantial increase of support for science and technology. The increase will amount to more than 50 per cent in this next five years. Mrs Gandhi, always sympathetic to science, has accepted a proposal put to her by Dr M. S. Swaminathan which is intended to strengthen government and university laboratories and to improve the quality of research.

Two new technology boards set up by the Indian government could ease the problem of high unemployment among India's scientific and technical personnel and give a new thrust to biotechnology research in the country.

A National Science and Technology Entrepreneurship Board is to be set up which will seek institutional credit for the 300,000 such unemployed persons to enable them to set up self-employed units. Surveys will be conducted to identify the areas in the country with development potential requiring scientific and technical support.

While some areas have a surplus in scientific and technical manpower (mainly due to unemployed polytechnic graduates or postgraduates) other areas are experiencing critical shortages. For instance, although there are about 4,000 vacancies for teachers in polytechnics in the country, suitable candidates are not available.

The Indian government has also decided to set up a biotechnology board to coordinate the functioning of biotechnology organizations in the country. Its purpose will be to utilize new concepts in biotechnology in agriculture, industry and medicine.

Mrs Gandhi said at the National Science Congress, India's biggest annual gathering of scientists, held in the southern city of Mysore on 3 January, that biotechnology was a frontier area of science, and that the country must take advantage of the latest technologies which could enable India to bridge the intermediate stages.

Sunil Saraf

CORRESPONDENCE

Referees unmasked?

SIR — The present climate of open decision-making in science has been reflected in the printing of signed editorials in *British Medical Journal* (3 October 1981). Surely now is the time to consider alternatives to the traditional policy of anonymity for the referees of learned journals.

The problems which can arise from such a policy have disturbed authors for many years. Recently (*Nature* 25 June 1981, p.608), it has been suggested that the disclosure of the names of the referees to authors would avoid corruption and injustices. The alternative, that the names of authors and institutions be withheld from referees (*Nature* 30 July 1981, p.402), has obvious flaws and double-blind evaluation (*Nature* 17 September 1981, p.293) is unlikely to be attained.

The publication of the names of the referees would give recognition to their responsibility and we feel would lead to an improvement in the standard of published work. At best, a referee can make a valuable contribution to a manuscript, which deserves open acknowledgement in the published paper. Conversely the cursory examination of research work has resulted in the growth in number of papers of dubious merit, despite editorial vigilance. Responsibility for the appearance of such publications should be shared by the referees and their names no longer concealed.

D.J. LEA
H. SHEPPEARD

Robert Jones & Agnes Hunt
Orthopaedic Hospital,
Oswestry, UK

Migration fund?

SIR — What practical steps are we going to take to aid our Russian colleagues who are not allowed to leave their country (see letter from Dikii *et al.*, *Nature* 24/31 December 1981, p.688)? Perhaps we should look more closely at why the Soviet government refuses to let them leave; one feels that there must be some governmental reason for the actions that are taken — it cannot, one hopes, be simply a whim of some highly placed individual.

The Soviet government could argue that it has invested heavily in the education of scientists and cannot afford to waste this investment. But the response to an application to emigrate is the withdrawal of scientific employment and the investment is lost anyway. Perhaps the Soviet government sees the use of this scientific education in another country as a double loss, and opts for what it sees as the better of two bad choices.

Interested governments, involved in scientific migrations, might press UNESCO or some similar body (the International Council of Scientific Unions perhaps) to set up a "Migrant Scientists Fund". This hard currency fund would be used to alleviate some of the injustices caused by scientific migration. For example, a country which loses a scientist could receive monetary compensation; the country to which he goes, which at the moment receives a gift of all that expensive

education and training, would pay into the fund. This would benefit many countries, in particular the poorer countries which scientists tend to leave, and should prove no hardship to the rich countries to which they go. New Zealand attracts qualified people, but also loses quite a number; on balance we would probably be drawing from the fund. Australia would contribute.

In the case of the Soviet Union the flow is essentially one way, immigration to the country is on a very small scale. The fund could recompense the Soviet government for its lost educational investment and this might be a welcome source of hard currency. Countries which wish to encourage the movement of scientists could actually donate to the fund.

IAN SMALLEY

New Zealand Soil Bureau,
Lower Hutt, New Zealand

Vide the Latin

SIR — In the leading article, page 389 in your issue of 3 December 1981, entitled "Tolerance but not quarter for creationists", it is asserted that "creationism is not a part of science but an alternative to it".

As the word science is derived from the Latin *scire*, to know, would it not have been nearer the truth to apply this statement to the theory of evolution? While making no claim to vast knowledge of things existing, as I consider such known things as the cochlea, or the lens of the eye, or three-dimensional perception, I find creationism truly essential to science. A great mind expressed this in *Romans* Ch. 1 v. 20: "The invisible things of God from the creation of the world are clearly seen, being understood by the things that are made, even his eternal power and Godhead."

D.H.T. CONWAY

Weymouth, Dorset, UK

Areas of neglect

SIR — I have been following with great and increasing interest the profound cogitations on creationism in your respected columns.

However, it is a pity that these discussions have been restricted to a particular field of biology. Surely there are many other sciences that need a thorough overhaul to put them into agreement with divine revelation. Take astronomy for example. It is high time that the godless Newtonian theories should be expelled and the Earth returned to its lawful place at the centre of the Universe.

Another science that is singularly deficient is psychology. Modern textbooks never mention the well-documented scientific facts of manipulation of the human mind by devils. Surely some enlightened state administrators should provide the necessary funds for an experimental investigation of these important phenomena. In this respect we have fallen far behind what was known in the Middle Ages, when at least a number of practical and efficient methods were in use for dealing with Satanic influences.

As a final example one could cite acoustics.

The most important application of this branch of physics — the demolition of city walls by sound waves — is no longer a fashionable subject of study, although new findings in the field of surface acoustic waves could provide a solution to this problem. It is a sad reflection on the state of present-day science that students never hear of it, except through a well-known negro spiritual.

S.V. VAECK

Brussels, Belgium

DNA and Europe

SIR — The article by Jasper Becker on the EEC safety dispute concerning recombinant DNA (*Nature* 24/31 December 1981, p.686) gives a quite correct picture of the situation. But Jasper Becker states that the socialists are in favour of a community directive. This is not quite correct. The socialist group in the European parliament has never voted on this question. Only the socialist members of the environment committee have given their opinion. Other members of the group, like me, have a different position for a multitude of reasons. In my view, only industrial and agricultural applications of recombinant DNA work should be regulated at an EEC level.

FRITZ GAUTIER (MEP)

Socialist Group,
Braunschweig/Brussels, Belgium

Brief encounter

SIR — In your summary "Hoyle on Evolution" (*Nature* 12 November 1981, p.105) you cite Hoyle as saying that for higher life forms to have emerged by chance is comparable to the chance that a tornado sweeping through a junk-yard will assemble the material into a Boeing 747.

This reminds me of a debate I had some years ago with Dr Douane Gish of the San Diego Institute of Creation Research. The occasion was a symposium in Utrecht organized by the Evangelische Omroep, the Dutch Evangelical Broadcasting Company. This company broadcasts the creationist message for several hours per week to Dutch audiences via television and radio. At the symposium in Utrecht I defended the theory of biological evolution, and Dr Gish defended the biological aspects of creationism. During the discussion Dr Gish remarked that the chance that amino acids would spontaneously form biologically active protein molecules was practically zero. I then said: "Dr Gish, if somebody had asked me on the day of my birth to calculate the chance that one day I would have a debate in Utrecht with Dr Gish from San Diego, I would have answered that this chance was practically zero. Am I therefore to understand that you are not Dr Gish from San Diego?" But my opponent had the perfect answer to that one. He said: "I am not here by chance, I was invited!"

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NEWS AND VIEWS

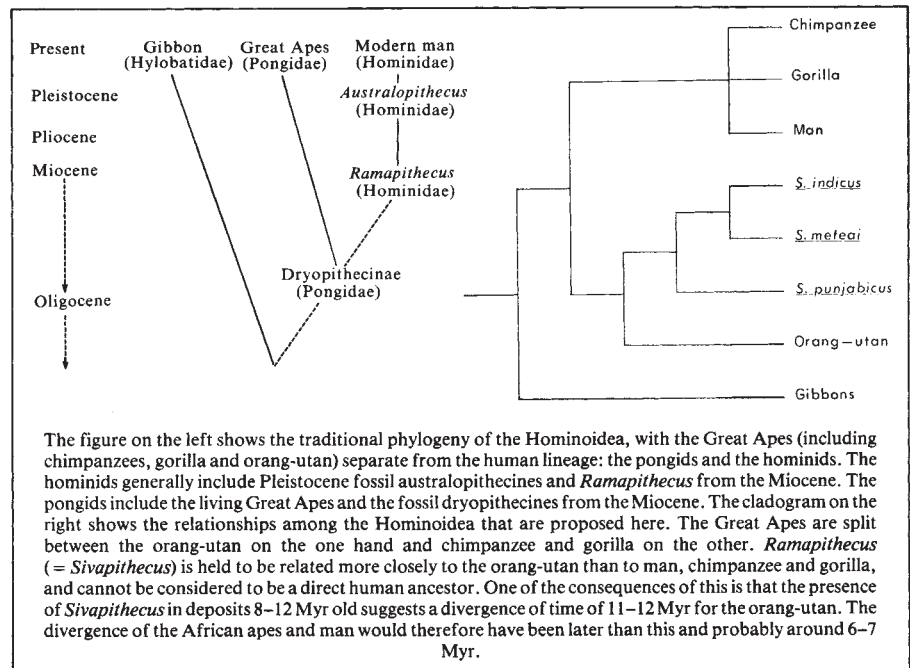
Hominoid evolution

from Peter Andrews

In the debate over human origins it has become the traditional view that *Ramapithecus* is the earliest known hominid. Set against this is the opinion, held by a minority, that *Ramapithecus* is more closely related to the orang-utan and is not thus directly connected with the evolutionary line leading to humans. The latter view receives fresh support in this issue of *Nature* (p.232) with the description of a new skull of *Sivapithecus* from Pakistan by David Pilbeam. The specimen is by far the most complete *Sivapithecus* yet found, and it provides information that was previously either unknown or based on reconstructions from less complete material. It will be examined here in relation to two important issues — the position of the orang-utan in relation to other living hominoids and the relevance of fossil primates, in particular *Sivapithecus* and *Ramapithecus*, to hominoid evolution.

The relationships of the living hominoid primates — apes and man — have been greatly clarified by the biomolecular work initiated nearly twenty years ago by Morris Goodman. Three cladogenic events in hominoid evolution have been recognized: the divergence of the gibbon from the common hominoid stock; the subsequent divergence of the orang-utan from the Great Apes and man stock; and the most recent divergence between man and the African apes. As well as defining the sequence of events, molecular anthropology has also been used to date these events, using the so-called 'molecular clock'. Many uses of the clock have been naive, but present estimates for the three events are: gibbon divergence 12 ± 3 Myr; orang-utan divergence 10 ± 3 Myr; and man – African ape divergence 6 ± 3 Myr (Andrews and Cronin, in preparation).

Two consequences arise from these results. First, there is no 'missing link' between apes and man. Man and African apes share a common ancestor, and the man–chimpanzee–gorilla clade shares a common ancestor with the orang-utan. The similarities in grade (mostly from shared primitive characters) between the orang-utan and the African apes means that they are superficially similar and that their



ancestral states would also be rather similar. They would therefore be correspondingly hard to identify in the fossil record, and a fossil like *Ramapithecus* might have similarities with either or both. Second, the molecular evidence suggests that the cladogenic events in hominoid evolution occurred later than many palaeontologists have expected. With *Ramapithecus* and *Sivapithecus* thought to be alive between 8 and 12 Myr ago, for example, the fossil taxa would appear to have arisen too early to be ancestral to humans.

The relationships of *Ramapithecus* and *Sivapithecus* can now be examined in more detail to see where their affinities lie. It is becoming apparent that the differences between the two genera are not as great as had formerly been thought. Greenfield (*Am. J. phys. Anthropol.* 50, 527; 1979) recently proposed that *Ramapithecus* is the same as *Sivapithecus*, and although his systematic procedure is suspect, there is a very high degree of similarity between *Sivapithecus* and *Ramapithecus*. Kay (*Int. J. Primat.*, in the press) has now made a much better case and suggests that *Ramapithecus punjabicus* become *Sivapithecus sivalensis*. Another alternative, which I prefer, is to retain *punjabicus* as a species of *Sivapithecus*. Therefore, in considering the relationships

of what was formerly *Ramapithecus* or *Sivapithecus*, the whole complex of *Sivapithecus* species must be considered. At the most conservative estimate there are at least four species of *Sivapithecus* found from 8 to 12 Myr and occurring from Greece and Turkey in the west to southern China in the east.

The specimen of *Sivapithecus* described in this issue of *Nature* is an adult *Sivapithecus indicus* with large parts of the face and palate preserved. It was badly broken when it was found, and Horace French and Rizwana Raza of Yale University have done a magnificent job in restoring it to its present condition. The specimen is the outcome of years of painstaking collecting in Pakistan by the Yale research project, directed by David Pilbeam, now at Harvard, and S.M.I. Shah in Pakistan.

Pilbeam has been very cautious in his assessment of the similarities his new specimen shows with the orang-utan. I will be less cautious and will list some of them: orbits higher than broad, poor development of the supra-orbital torus, narrow interorbital distance, smooth and unstepped floor of the nasal cavity, large zygomatic foramina set above the lower rim of the orbit, extremely small incisive foramina, narrow and slit-like palatine foramina, thick enamel and low cusp

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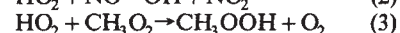
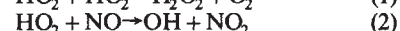
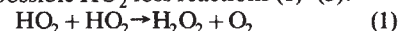
profile on the molars and very large incisors in comparison with the lateral incisors. All these characters are present on the *S. indicus* skull, and some at least are known to be present on other species such as *Sivapithecus metei* and *Sivapithecus punjabicus*. They are all interpreted as being derived from the primitive hominoid condition and are thus specializations shared with the orang-utan. There are also many primitive characters shared with the orang-utan, such as the poor development of the supraorbital torus and the slope of the nose, and other characters like the great depth of the zygomatic bone that show greatest resemblance to the orang-utan but are within the limits of variation of other hominoid species. Set against this list of characters are a few which show similarities with man. Of particular importance are the robustly built jaws, the lateral rotation of the canines and possibly low canine sexual dimorphism. Another similarity with man, the thick enamel and

low cusp relief of the molars, is also shared with the orang-utan and cannot be considered diagnostic. The similarities with man are fewer in number and more strongly inter-related with each other than are the similarities with the orang-utan, and it is my belief that *Sivapithecus* must be seen as a relative of the orang-utan. It is shown thus on the accompanying cladogram.

It thus appears that *Sivapithecus* (including '*Ramapithecus*') is part of the orang-utan clade, and both are more distantly related to man than are the African apes. In other words '*Ramapithecus*' can no longer be considered as part of the human lineage but as part of the orang-utan lineage. The recognition of this relationship provides fossil evidence for the divergence date of the orang-utan, and the time span of 8–12 Myr for the species of *Sivapithecus* is certainly compatible with the molecular date of 10 ± 3 Myr. □

2×10^6 molecules per cm^3 is estimated from our measurements. Mid free-tropospheric (that is, 5–6 km) values over the above latitude range were typically a factor of two to three lower than those observed in the marine boundary layer. These OH values are significantly higher than the globally averaged value estimated by several investigators based on Northern/Southern Hemisphere CH_3CCl_3 measurements^{9–11}. This result is consistent, however, with the higher than average levels of H_2O and UV actinic radiation that normally characterize the above latitude zones. They further suggest that in the tropical marine boundary layer, OH radicals are very likely to be responsible for initiating the oxidation of numerous compounds released into the atmosphere in reduced oxidation states. Approximate photochemical lifetimes based on oxidation by OH at a level of 2×10^6 molecules per cm^3 are SO_2 (3.5 days), CH_3SCH_3 (1 day), H_2S (1 day), NO_2 (0.7 day), C_2H_4 (0.7 day), C_3H_8 (0.2 day), CO (20 days) and CH_3Cl (124 days). Virtually all these lifetimes are much shorter than those predicted from washout/rainout or dry deposition.

A final point concerns tropical marine tropospheric-photochemical activity and its relation to the GAMETAG observations which showed no direct evidence of any photochemical generation of O_3 . In spite of this observation, it is important to understand that the absence of O_3 generation cannot be equated with the absence of free radical chemistry or the lack of importance of photochemistry, in general. It instead suggests that of the possible HO_2 loss reactions (1)–(3):



reaction (2), under tropical marine boundary layer conditions, is unsuccessful in competing with reactions (1) and (3). This result is a direct consequence of the previously described low levels of NO in the tropical marine boundary layer. It should be stressed, however, that the absence of O_3 production does not preclude the use of marine tropical areas of the world as future field sampling sites for studying free-radical tropospheric chemistry. In fact, the simplified chemistry of this region, resulting from highly reduced levels of hydrocarbons and their oxygenated analogues, should present the best opportunity for unravelling many key aspects of tropospheric chemistry. □

Measuring atmospheric gases and aerosols

from D.D. Davis, W.L. Chameides and C.S. Kiang

SOME nine months ago several of the findings of the National Science Foundation-supported Global Atmospheric Measurements on Tropospheric Aerosols and Gases programme (GAMETAG) were critically reviewed in these columns¹. As part of the planning group for this field sampling programme, we welcome efforts to bring GAMETAG results before a larger spectrum of the scientific community. We would now like to go on to clarify some points that were made earlier and to add new information from the most recently available results.

The first point involves the NO/ HNO_3 GAMETAG measurements. Before the GAMETAG flights, the little information available on NO_x in remote regions of the troposphere^{2–6} suggested that NO levels were in the 100–1,000 p.p.t.v. (parts per 10^{12} by volume) range. The GAMETAG NO instrument, which had a lower detection limit of 50–60 p.p.t.v., gave readings which indicated that over thousands of miles of continental wilderness area and over large stretches of the open Pacific Ocean, NO levels were typically below that lower detection limit. The observations⁷ are the first to indicate that NO_x levels in remote marine and northern continental tropospheric air are much lower than previously believed. Subsequently, MacFarland *et al.*⁸ reported that NO levels in a limited region of the

equatorial Pacific were in the 1–10 p.p.t.v. range but the GAMETAG HNO_3 observations were the first extensive remote-global tropospheric data base for this species. Of particular importance is the observation that lower levels of HNO_3 are found in the marine boundary layer than in the middle free troposphere over extensive regions of the open Pacific Ocean. The results are in direct contradiction with all earlier predictions of NO_x/NO_y altitude profiles and show the need for significant revisions in the tropospheric nitrogen budget.

A second point involves new data now available on the question of photochemical activity over tropical and sub-tropical marine areas. Since Penkett wrote¹, the data have been released in manuscript form and are shortly to appear in print. Direct OH observations show tropical mid-day marine boundary layer OH levels ranging from $\sim 2 \times 10^6$ up to a high of 1.5×10^7 molecules per cm^3 . It is argued by some that the absolute accuracy of the OH system may be in error by as much as a factor of two (the authors think this unlikely) but even if this were so and OH levels were assigned a value a factor of two lower, the conclusion that OH levels in the tropical marine boundary layer are typically quite high would still be clearly justified. It is estimated that the above observations imply a diurnally and seasonally averaged OH value of $\sim 2 \times 10^6$ molecules per cm^3 for the tropical marine boundary layer. For spring and summer conditions, at sub-tropical latitudes, a similar diurnally averaged OH value of

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A hydrothermal plume remobilizes sedimentary organic matter

from Andrew S. Mackenzie

In this issue of *Nature*, Simoneit and Lonsdale¹ describe petroleum found in dredge samples containing hydrothermal minerals, taken near a hydrothermal vent in the Guayamas Basin, Gulf of California. This 'hydrothermal petroleum' has a quite different composition from 'commercial petroleum' and its distinctive features are related to the rather special circumstances of its generation and migration. There is no evidence that the hydrothermal petroleum has a deep-seated origin and has emanated directly from the vent. Instead, the results suggest that part of the biologically derived organic matter of sediments in the neighbourhood of the vent can be remobilized and transported by the hydrothermal plume.

The most striking feature of the hydrothermal petroleum is its chemical resemblance to the organic matter which can be extracted, with common organic solvents, from relatively immature sedimentary rocks. Indeed, in one sample, a number of organic molecules, whose carbon skeletons have unambiguous links with known biological molecules, are still present as their less stable forms. Within this immaturity, the authors have detected

certain components whose continued presence in large relative abundances is best explained by a relatively short-lived heating at temperatures well above those normally found in connection with petroleum formation. The evidence can best be accommodated in a model where the immature organic matter of the Quaternary sediments around the hydrothermal vent has come into contact with a hot hydrothermal plume and has then rapidly cooled as a result of migration in the hydrothermal flow and condensed at the seabed.

Organic geochemistry is much concerned with specific problems of petroleum geology, particularly those associated with thermal maturation². The present paper¹ and others from the same laboratory³ show some of the advantages of being able to study Earth processes at a specific organic molecular level and perhaps indicate the further contributions the field is poised to make in many other areas of the Earth sciences. Although a tremendous amount has been achieved in cataloguing a large number of specific but previously unknown chemical structures of the organic compounds occurring in sediments and petroleum⁴, most of the applications to date have been very empirical. Simoneit and Lonsdale have made use of the sciences of chemical

kinetics and thermodynamics in a descriptive sense to predict how the hydrothermal petroleum was formed. Large relative abundances of peri-condensed aromatic hydrocarbons and steroidal alkanes are said to reflect relatively short-lived contact with high temperatures (certainly greater than 250°C), because other reactions, which destroy the aromatic hydrocarbons⁵, add hydrogen to triterpenoid alkenes⁶ and isomerize triterpenoid and steroidal alkanes⁷⁻⁹, have been promoted to a lesser extent. This observation implies that the rates of the former types of reaction are accelerated to a greater extent by a major increase in temperature than those of the latter reaction types, and stems from the principles of chemical kinetics.

The chemical composition of the migrated hydrothermal petroleum also shows specific differences from organic matter found close to igneous sills³. Unsaturated hydrocarbons (alkenes) with carbon-carbon double bonds in the middle of the carbon chains occur in both environments, whilst those with double bonds at the end of the carbon chains are restricted to the organic matter next to sills. Simoneit and Lonsdale argue that the mid-chain alkenes can survive the migration in hot fluids, because of their higher thermodynamic stability, whereas the less stable terminal alkenes are completely destroyed.

Such descriptive integrations of physical chemistry into molecular organic geochemistry are welcome and valid. In the future, attempts will be made to derive more mathematical expressions for organic geochemical processes, particularly for those which can now be studied at a molecular level. Universally applicable expressions are more likely to emerge from the relatively simple interconversions between two known molecular structures than from the complex physicochemical processes which generate petroleum. These expressions will permit a more quantitative evaluation (what temperature and for how long) of the past geological events which have affected the organic mixtures being studied. In such a form molecular organic geochemistry will provide a new set of tools to Earth scientists. □

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The Thar.

100 years ago From *Nature* 25, 26 January, 297, 1882.

THE THAR (*Capra jemiaica*). — The peculiar Himalayan Goat, known to the Indian sportsmen as the Têhr, Thar, or Tahir, was first described in 1828 by Hamilton Smith, and named *Capra jemiaica*, from the district of Jemlab, to the north of Nepaul, in which his specimen was procured. It is found, however, as Dr Jerden tells us, throughout "the whole extent of the Himalayas at great elevations, generally above the limits of forest and not far from the snow. It frequents rocky valleys and very steep and precipitous ground, and is often seen perched on what appear to be inaccessible crags. If alarmed whilst feeding, these animals all go off at full speed with a clattering sound, but soon halt and turn to gaze on the intruder."

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Prostaglandins and cancer

from Peter Alexander

PROSTAGLANDINS, thromboxanes, prostacyclins and leukotrienes are produced by most tissues of the body by oxidation of arachidonic acid (AA). Their possible importance in cancer, at levels including carcinogenesis, the rate of proliferation of the tumours and the interaction between the tumour and the host, was the subject of a recent conference*.

Their significance can be seen in relation to the possibility that cancer may be prevented not only by withdrawing carcinogenic stimuli but also by intervening in the complex sequence of events between exposure to a carcinogen and the occurrence of a malignant neoplasm. Prostaglandin (PG) synthetase inhibitors, like aspirin, may be candidates for the 'chemoprevention' of cancer. Most chemical carcinogens need to be converted by metabolic processes into a reactive carcinogenic substance which can combine in a covalent chemical reaction with intracellular nucleophilic constituents. This metabolic activation can occur by reduction and by hydrolysis but most commonly requires oxidation. The cytochrome P450 monooxygenase systems have been shown to be an important oxidative pathway for the production of proximate carcinogens, and evidence is now accumulating that such oxidation can also occur during prostaglandin synthesis.

L. Marnett (Wayne State University, Detroit) showed that in the reactions involving benzopyrene, the conversion of the pro- to the proximate carcinogen was brought about by by-products (free radicals) produced during the synthesis of prostaglandins, although a direct role of lipoxygenase could not be totally excluded. With some tissue preparations, like ribosomes from ram seminal vesicles, an aspirin-like drug totally blocked the production of the proximate carcinogen, whereas with intact epithelial cells the active metabolite was produced via both the P450 and the prostaglandin pathway (T. Eling, National Institute of Environmental Research, North Carolina). Before inhibitors of AA metabolism can be seriously considered for chemoprevention, the relative contribution of prostaglandin-related oxidations in the formation of carcinogens must be determined in different organs. T. Zenzer (V.A. Medical Center, St Louis) presented early results from continuing *in vivo* studies with the potent bladder

carcinogen FANFT (a 5-NO₂ furan derivative), the oxidative metabolite of which binds to DNA *in vivo*. Co-administration of aspirin prevents both binding to DNA and the appearance of early hyperplastic and possibly pre-cancerous lesions in the bladder. It will be another 12 months or so before it is known whether the incidence of bladder carcinomas in rodents has been reduced.

In many experimental systems carcinogenesis can be separated into two processes — initiation by the chemical reactive carcinogens and promotion. The most effective and widely studied promoting agents are phorbol esters such as TPA. The biochemical basis of promotion remains unclear because these agents induce a great many changes in cells and tissues. Prostaglandins and related substances could be involved as TPA increases release of their precursor, AA, from cell membranes and this produces an increase in cellular prostaglandins (E. Bresnick, University of Vermont). Indeed, this may contribute to the intense inflammation produced by all the phorbol esters that are active as promoters. R. Boutwell (University of Wisconsin) considers that elevation in ornithine decarboxylase activity is a key event in promotion and this is reduced if inhibitors of PG synthetase are given at the same time as TPA. The decisive test, however, is whether tumour promotion by TPA can be prevented *in vivo* by modulating prostaglandins. G. Fürstenberger finds that indomethacin, a potent prostaglandin synthesis inhibitor, prevents promotion by TPA of skin tumours following initiation with a polycyclic hydrocarbon and that administration of PGF_{2α} reverses this inhibition. But S. Fischer (Oak Ridge National Laboratory) is cautious about simplistic interpretations because she finds that low doses of indomethacin actually enhance promotion and that interference with promotion by high doses may not be due to inhibition of prostaglandin synthesis. She considers, however, that products from AA obtained by the lipoxygenase pathway may be concerned with promotion.

There is mounting evidence for a role of products of the AA cascade in the growth and spread of tumours once they have arisen. *In vitro* there are correlations between growth rate and intracellular prostaglandin concentrations which are not easy to interpret. Certain prostaglandins, notably PGE, slow cell division (B. Jaffe, State University of New York, Brooklyn, and S. Hammarstrom, Karolinska Institute), while L.J. de Asua (Friedrich Miescher Institut) demonstrated that PGF_{2α} is highly specific in causing cells rendered quiescent, for example by serum

deprivation, to initiate DNA synthesis, probably by causing changes in the cytoskeleton. It is not, therefore, surprising that experiments in which inhibitors of prostaglandin synthesis are added to cell cultures or given *in vivo* to tumour-bearing animals give rise to conflicting results. In many respects the most impressive effects of prostaglandins on the growth of cells *in vitro* is the induction of maturation and their possible involvement in retinoid-induced differentiation. PGE, probably produced by monocytes, regulates proliferation and differentiation of stem cells in the bone marrow both *in vivo* and *in vitro* (R. Bockman, Memorial Sloan Kettering Cancer Center, New York). The observation that, *in vitro*, certain prostaglandins, notably PGA and PGI₂, can induce differentiation of myeloid (T. Breitman, NIH) and erythroid (G. Santarro, Ospedale Ruinetti, Rome) leukaemia cells as well as of mouse mammary carcinoma (P. Rudland, Ludwig Institute, UK) and neuroblastoma (K. Prasad, University of Colorado Medical Center) cells, constitutes the best reason for persevering with attempts to inhibit the growth of tumours using prostaglandins.

It is also possible that prostaglandins may influence the interplay between the host and the tumour. A popular concept is that some prostaglandins depress immune responses and that inhibition of prostaglandin synthesis will enhance a putative immune reaction of the host to the tumour. As yet, there is little hard *in vivo* data in support for immunotherapy based on prostaglandins. However, there are non-immunological interactions between the tumour and the host in which prostaglandins are involved and which might be manipulated in a therapeutically beneficial manner. Investigation into the control of metastases to bone and of the hypercalcaemia associated with malignancy using inhibitors of prostaglandin synthesis has been motivated by the finding that some prostaglandins cause osteolysis and calcium release from bone *in vitro* and that in two animal models, hypercalcaemia is induced by tumours that make large amounts of prostaglandin and can be stopped by aspirin-like drugs. Unfortunately, in man there is no consistent correlation between serum levels of prostaglandin and hypercalcaemia nor will inhibition of prostaglandin synthesis effect bone metastases or hypercalcaemia (T. Powles, Institute of Cancer Research, Sutton, UK).

Of considerable interest are experiments by K.V. Honn (Wayne State University, Detroit) which are based on the premise that platelet aggregation favours the spread of tumours. Accordingly, he tested

*An international conference on 'Prostaglandins and Cancer' was held from August 30 to September 2 1981, in Washington DC, under the chairmanship of T.J. Powles (Royal Marsden Hospital, Surrey), R. Beckmann (Memorial Sloan-Kettering Cancer Center, New York), K.V. Honn (Wayne State University, Detroit) and P. Ramwell (Georgetown University School of Medicine, Washington DC).

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in an animal tumour system selective inhibitors of Tx, which promotes platelet aggregation, and the administration of PGI₂ (or of substances which facilitate vascular PGI₂ synthesis), which prevents the formation of platelet emboli. Both procedures reduced the incidence of lung metastasis and in addition slowed the growth of the tumour growing at the site of injection. This latter effect is unlikely to involve interference with platelet

aggregation and is thought to be a direct effect on the rate of proliferation of the tumour cells by increasing cyclic AMP.

The meeting was successful in bringing together cancer research workers and many of the leaders in the area of prostaglandins. It was encouraging to both groups that manipulation of the AA cascade may have a role in both prevention and treatment of cancer although the key experiments remain to be performed. □

A new British flower

from Peter D. Moore

BOTANISTS in the tropics will probably throw up their hands in horror but the fact remains that the discovery of a new species of flowering plant in the British Isles (and probably a native one at that) is news. Rix and Woods¹ have now officially recorded the finding of *Gagea bohemica* at Stanner Rocks, near Kington, mid Wales. Its inconspicuous leaves and flower undoubtedly contributed to its success in eluding detection until recently and to its initial misidentification. But the find also has some interesting biogeographical implications, for *G. bohemica* is a species of continental distribution, reaching its northwestern European limits in central Germany and the Seine Valley in France, and extending into central, eastern and southern parts of the European mainland. Stanner Rocks is a site far-removed from the remainder of its range.

One must consider the possibility that the plant has arrived at this location by chance dispersal but, as Carlquist² has recently emphasized, it is very difficult to study freak phenomena of this kind. *G. bohemica* reproduces by seed (though at Stanner Rocks only one per cent of the population flowers and no capsules have been observed) and by bulbils. Unlike some lake and seashore sites, a rock outcrop is an unlikely spot for the concentration of a bird migration route, making bird transport an unlikely explanation of its presence.

It is important to consider that Stanner Rocks boasts an unusual assemblage of rare plants currently exhibiting disjunct distributions, including *Lychnis viscaria*, *Veronica spicata* and *Scleranthus perennis*. Also, on some neighbouring outcrops in mid Wales, is *Potentilla rupestris*³. All of these have continental distributions, though *L. viscaria* and *S. perennis* extend further north than the others.

All these species are plants of low growth habit and demand light. Pigott and Walters⁴ proposed that plant assemblages

having a number of open habitat species of generally disjunct distribution were often associated with disturbed or unstable sites in which the forest cover had remained incomplete throughout post-glacial time. The plants now isolated in such sites may represent relicts of the vegetation of late-glacial or early post-glacial times, which have been extinguished over the bulk of their former range by forest expansion and consequent shading. In the case of Stanner



Gagea bohemica

Rocks, such an explanation is supported by the sub-fossil record of certain of the plants concerned. *S. perennis* pollen has been found in late-glacial sediments of mid Wales⁵ and a seed of *L. viscaria* was found by Dickson, Dickson and Mitchell⁶ in late-Devensian material from the Isle of Man. Unfortunately, no sub-fossil records are available for *V. spicata*, *P. rupestris* or *G. bohemica* in Britain, but their current, rather southerly, continental distributions do not make them likely candidates for late-glacial relicts.

Lamb⁷ has constructed global maps of probable atmospheric pressure systems during winter and summer at several stages of post-glacial history, and he concludes

that the early part of the post-glacial in north-west Europe (pre 7,000 BP) was characterized by a vigorous circulation of air masses which would have brought dry, anticyclonic conditions to the area both in winter and summer. It may well be that many southern continental plant species were able to migrate into what are now oceanic regions in the north and west during those times. This would have brought into contact a variety of continental species of somewhat southerly and northerly affinities; it is interesting to note that all five of the species under discussion here are currently found together in central Poland, which may give some indication of the climatic conditions during the period of mixing. Rix and Woods¹ note that the Stanner Rocks specimens of *G. bohemica* are morphologically most akin to the western French populations on the continent, which may suggest a west European origin for the British population of this species.

The subsequent history of the mid Wales area has involved forest spread and increasing oceanicity of climate, both of which will have operated against the survival of all these species; but the rock outcrops, such as Stanner, have evidently acted as refuges. Such outcrops would not only have provided steep, unstable slopes keeping the woodland and canopy open and discontinuous, but may also have created microclimatic conditions appropriate for the survival of continental species of plant. *G. bohemica* on Stanner Rocks is currently restricted to shallow soils on southern and eastern aspects. These locations would be well insulated in summer, providing a 'micro-continental' climate, rather analogous to some of the present-day East Anglian breckland habitats of *S. perennis* and *V. spicata*. The importance of aspect for survival of a species at the edge of its climatic range is well documented for such plants as *Cirsium acaule*⁸ which reaches its northern British limit in Derbyshire but survives there mainly on southerly aspects. Even under these conditions fruiting is poor and experiments involving daily spraying of the developing fruit with water, thus lowering overall temperatures, reduced success even further. *G. bohemica* fails to fruit at Stanner, but persists by vegetative propagation.

The record of *G. bohemica* from Stanner Rocks is thus more than just one more species on our list — it may also provide important evidence of the climatic and vegetational history of the British Isles. □

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Analysing atmospheric carbon dioxide levels

from Michael J. Rycroft

THE possibility that changes in climate may be brought about by increasing levels of carbon dioxide in the atmosphere made a recent conference* devoted to the analysis and interpretation of atmospheric CO₂ data of particular significance. The CO₂ increase is caused primarily by the burning of fossil fuels and secondarily by deforestation in the tropics and changing agricultural practices. Very recently such problems have been put in context by Bach *et al.*¹ and Kellogg and Schwart², and discussed by Kandel³. Following the pioneering work of Manabe and Wetherald⁴, several complicated model calculations⁵⁻¹⁰ have been performed; these have been reviewed by Schiff¹¹.

For a doubling of the CO₂ content of the atmosphere, these models calculate that the global mean surface air temperature would rise by 2–4 deg, but with a larger increase at high latitudes¹². It has been suggested that this could lead, within a thousand years, to deglaciation of the West Antarctic ice sheet^{13,14}. The consequent rise of the mean sea level^{15,16} would have a profound effect as population is concentrated in low-lying areas of the world. Associated with the temperature rise would be changes of atmospheric circulation and the spatial distribution of rainfall. The changed soil moisture patterns could have serious implications for agriculture and food production. The study of atmospheric CO₂ is thus closely linked with other biogeochemical processes, studies of carbon budgets and the global carbon cycle¹⁷, the biogeochemical cycles of nitrogen, phosphorus and sulphur, and their mutual interactions¹⁸ in the atmosphere and oceans¹⁹.

R. M. Rotty (Institute for Energy Analysis, Oak Ridge Associated Universities, US) has shown earlier that the global production of anthropogenic CO₂ by fossil fuel burning (98%) and cement manufacture (2%) has increased exponentially between 1860 and 1914 and since 1945. Simple exponential growth, with an *e*-folding time of 24 years, corresponds to compound interest at 4.3% per year from a pre-Industrial Revolution value of at most 290 parts per million (p.p.m.) by volume. However, the situation is not so simple. From 1950 to 1973, the growth rate of CO₂ emission into the atmosphere was 4.6%, and since 1973, when OPEC increased oil prices dramatically, the growth rate halved to 2.3%. At present, 5.3 Gigatonnes (5.3 ×

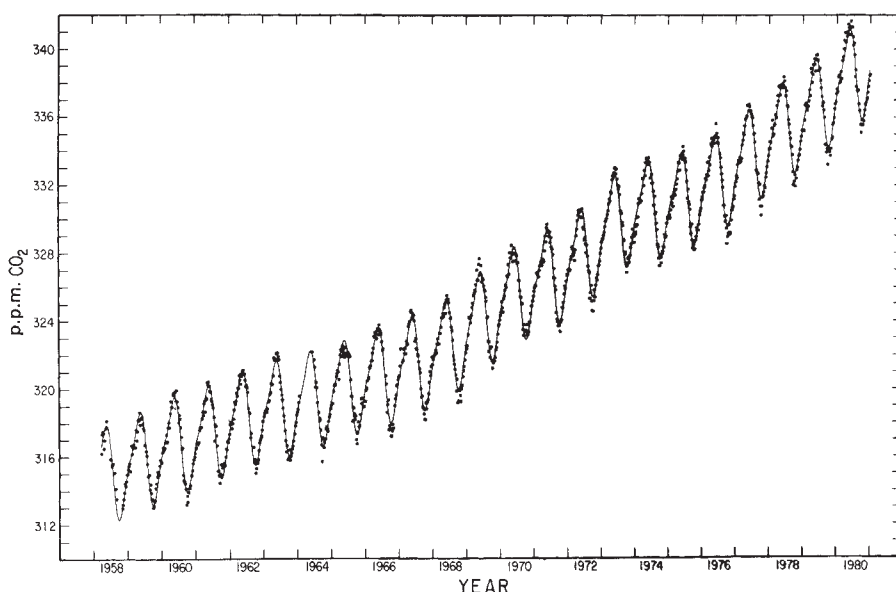


Fig. 1 Atmospheric CO₂ concentrations (weekly averages) measured at Mauna Loa, Hawaii (courtesy of C. D. Keeling). The curve is a spline fit assuming an annual variation of constant amplitude superposed on a long-term trend.

10¹² kg) of carbon are being released per year into the atmosphere as CO₂ (19.4 Gt per year). As this adds to the 700 Gt of carbon already present in the atmosphere as CO₂, it would seem that the CO₂ content of the atmosphere should, at the present time, simply increase by 0.76% per year. With a typical atmospheric CO₂ concentration of 335 p.p.m., it might be expected that the concentration of atmospheric CO₂ should increase by 2.5 p.p.m. per year.

However, C. D. Keeling (Scripps Institution of Oceanography) presented measurements of atmospheric CO₂ concentrations made since 1958 using a non-dispersive IR absorption spectrometer at Mauna Loa Observatory, Hawaii, at 20°N. Each point in Fig. 1 is a weekly average; the annual variation is explained by seasonal changes of the rates of photosynthesis and respiration by plants and the soil. Last year, the 340 p.p.m. value was exceeded for the first time. Also evident in the figure is the long-term, quasi-exponential increase of CO₂ concentration, at a rate of 1.3 p.p.m. per year, or 0.40% per year. Thus only 53% of the increased CO₂ is retained in the atmosphere; this is termed the airborne fraction. The remaining 47%, or excess fraction, is taken up mainly by the oceans at middle and high latitudes. Quantitative studies of the air-sea interaction are difficult; comparisons between modelled and observed results are complicated by the fact that the time scales of oceanic CO₂ uptake are between 10 and 100 years (at

lesser to greater depths), and are of the same order as the 24 year time constant of the atmospheric CO₂ increase.

Of the CO₂ production, R. M. Rotty showed that 91% occurs at latitudes between 30 and 60°N, primarily over land. This fact accounts, in part, for the CO₂ latitudinal gradient, shown as differences from the Mauna Loa mean in Fig. 2, and derived from measurements made at different observatories and aboard ships and aircraft. Additional factors are the CO₂ source associated with the warm sea surface in equatorial regions and the relatively slow interhemispheric mixing of atmospheric CO₂ across the Inter-Tropical Convergence Zone. CO₂ concentration differences of 1 p.p.m. can be accounted for by the interhemispheric CO₂ fluxes of

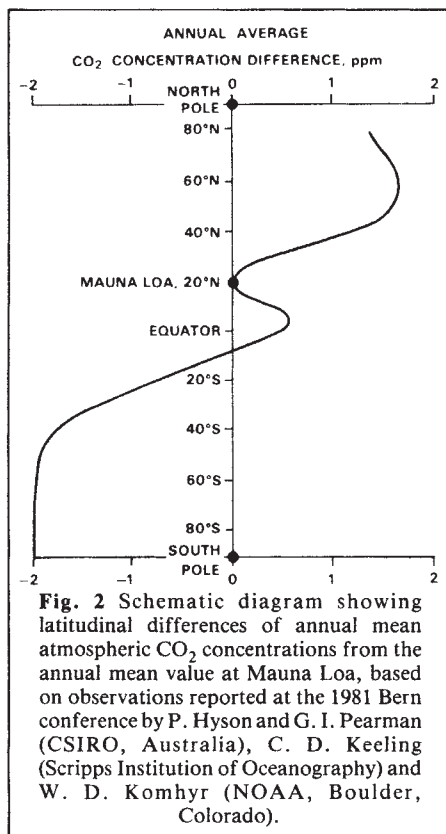
* The joint World Meteorological Organization, International Council for Scientific Unions and United Nations Environment Programme sponsored conference on 'Analysis and Interpretation of Atmospheric CO₂ Data' was held at the University of Bern, Switzerland, from 14 to 18 September 1981, with support from the International Association of Meteorology and Atmospheric Physics and the US Department of Energy, and was attended by seventy scientists from twenty countries. A book containing abstracts and some papers has been published by the WMO; it is planned that the complete set of papers presented at the conference will be published in the *Journal of Geophysical Research*.

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about 1 Gt per year.

The largest annual variations of CO_2 concentration, with a difference of 15 p.p.m. between maximum and minimum values, occur between 50 and 70°N, there being a 6 p.p.m. difference at Mauna Loa and no annual variation between 10 and



20°S. There is also some evidence of two and four year periodicities. R. B. Bacastow and C. D. Keeling (Scripps Institution of Oceanography) have associated large increases in the CO_2 concentration with a minimum value of the Index of the Southern Oscillation of the atmosphere and with El Niño occurrences. These latter events — the presence of 2 deg warmer, nutrient-poor water in a region off the Peruvian coast at 80°W and out to 180°W, which is associated with a reduction, or even cessation, of the upwelling of colder, nutrient-rich water — are of great significance to the region's fishermen.

Evidence for slight decreases in the atmospheric $^{13}\text{C}/^{12}\text{C}$ ratio has been obtained from mass spectrometer analyses²⁰. Both $^{13}\text{C}/^{12}\text{C}$ and $^{14}\text{C}/^{12}\text{C}$ ratios in the cellulose of tree rings similarly reflect the ratios' atmospheric values during successive year's growing seasons. It is concluded that biospheric processes, both soil and plant, acted as a net source of atmospheric CO_2 from 1860 until about 1950; since then, biospheric sources and sinks of CO_2 have cancelled each other out. For example, the present deforestation in the tropics could be balanced by reforestation at middle latitudes. Also, ^{14}C in corals gives information on CO_2 uptake by the surface water of the oceans and on surface water circulation. Of course, the

radiocarbon concentrations of the atmosphere and ocean have risen considerably since the thermonuclear bomb tests of the 1950s.

To identify new trends, continuing and uninterrupted measurements of atmospheric CO_2 concentrations are required at up to 20 well distributed sites around the globe. The required absolute accuracy is ± 0.1 p.p.m.; this is achievable only by performing very careful inter-calibrations, using standard gases, between the different experimental groups. Further, there is a pressing need for

additional observational studies of seasonal and annual variations of atmospheric CO_2 at particularly interesting sites. Such observations, required to a relative accuracy of ± 0.1 p.p.m., can be interpreted in terms of sources and sinks of CO_2 , and should lead to an improved understanding of the carbon budget. With improved observational knowledge, and with the further development and validation of climate models, better predictions may then be made of the environment in which mankind will find itself in the coming decades and centuries. □

Mammary gland as an endocrine organ: implications for mastectomy

from Jared M. Diamond

THE traditional view of the mammary gland is that it receives many hormonal signals but sends none. Oxytocin acts on it to trigger milk ejection, prolactin to stimulate milk secretion, and oestrogens, progesterone, prolactin and growth hormone to stimulate gland growth during pregnancy. The only direct signals that the mammary gland sends to the rest of the body have been thought to be concerned with its own regulation — nerve impulses (the suckling reflex) cause prolactin and oxytocin release from the pituitary and reflexly control milk production. This traditional view has determined the assessment of the costs and benefits of mastectomy, an operation that has often been used to deal with mammary tumours. Although mastectomy has been increasingly criticized for its psychological consequences, it has at least been thought that the operation is without physiological consequences since the mammary gland has no role outside of lactation.

This dogma has been undermined by important recent studies¹⁻⁴ of Peaker (Hannah Research Institute, Ayr) and Maule Walker (ARC Institute of Animal Physiology, Babraham), demonstrating that, in goats, the mammary gland releases hormones into the general circulation and mastectomy causes major disturbances of other reproductive functions besides lactation. These observations clearly indicate the need for the study of possible physiological consequences of mastectomy in humans.

The roots of the new observations go back at least 75 years. In 1906 Marshall and Kirkness⁵, studying urinary sugars in mastectomized guinea pigs, briefly commented: "Considerable difficulty was experienced in inducing the guinea pigs to

breed after the removal of the [mammary] glands, some of them failing to do so although kept for over eight months in company with males, and in an apparently perfectly healthy condition". Four of these mastectomized guinea pigs did eventually become pregnant, resulting in one premature still-birth, one abortion and four normal births. In the late 1950s Linzell worked out procedures for autotransplanting mammary glands of goats, and in the following 20 years he used the improved experimental access provided by this preparation to answer many problems of mammary physiology. Early in these studies he noted⁶, in the course of a long paper on milk secretion, that loss of mammary tissue in goats was associated with increased incidence of several reproductive disorders: infertility, mastitis, abortion and maternal death at parturition. Peaker and Maule Walker have now followed up these observations by studying mastectomized goats, four of which have been followed through three pregnancies. The reproductive consequences of mastectomy in the first breeding season were a lengthened first oestrus (115 h vs 30 h in controls), shortened oestrous cycles (8–17 days vs 22–24 days in controls), reduced fertility and slightly shortened pregnancy. In the second pregnancy, and even more in the third pregnancy, there was peripheral oedema before term, premature onset of labour and prolonged labour with weak cervical contractions and no cervical dilation.

How does removal of the mammary gland lead to these reproductive disturbances? Several possible mechanisms involve disruption of lactation, which is known to have systemic effects. Nerve impulses from the mammary gland to brain during lactation stimulate reflex release of pituitary hormones that control not only milk production in mammals generally but also embryonic development in

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marsupials⁷. Lactational amenorrhoea may similarly involve mammary reflex control of pituitary hormones, in this case inhibition of follicle-stimulating hormone and luteinizing hormone release. Withdrawal of large quantities of glucose and other substrates for milk synthesis from the bloodstream leads to changes in levels of hormones that regulate blood concentration of these solutes, such as insulin and glucagon.

However, Peaker and Maule Walker observed no difference between lactating and non-lactating non-mastectomized goats in fertility or in oestrous cycle periodicity. Furthermore, the mastectomized goats exhibited oestrous disorders and impaired fertility before they had ever become pregnant or lactated. These facts suggest that interruption of lactation is not responsible for the reproductive disorders associated with mastectomy. A more likely explanation comes from new evidence that non-lactating mammary gland releases hormones into the systemic circulation, in addition to producing local hormones such as prostaglandins during lactation. From earlier observations by Linzell and colleagues⁸⁻¹⁰ that the mammary gland of

goat can metabolize steroid hormones, Maule Walker and Peaker¹ have gone on to find that the rise in plasma concentration of oestradiol-17 β during the last four days before term is due almost entirely to increased production of the hormone by mammary gland.

It remains to be seen whether reproductive disorders in mastectomized goats are related to this mammary production of oestradiol-17 β , or of other known steroid, peptide, or protein hormones, or of 'new hormones', or else to a non-hormonal mammary signal. The great variability among mammals in the control of reproduction suggests that the goat is not necessarily a universal model and studies on other mammalian species, especially on humans, are now urgently needed. $\square$

1. Maule Walker & Peaker *J. Physiol., Lond.* **284**, 71 (1978).
2. Peaker & Maule Walker *Nature* **284**, 165 (1980).
3. Maule Walker & Peaker *J. Physiol., Lond.* **312**, 63 (1980).
4. Peaker in *Hormones and Metabolism in Ruminants* (eds Forbes & Lomax) 122 (Agricultural Research Council, London, 1981).
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Exciting Rydberg atoms in cavities

from Peter Knight

HIGHLY excited atoms with one electron orbiting at large distances from an unexcited core are termed Rydberg atoms and have remarkable properties. Their size gives them a large polarizability, and they are easy to ionize in weak electric fields. If they possess a large angular momentum they do not return rapidly to their ground state by radiative decay simply because decay photons cannot easily carry away all that excess angular momentum. Rydberg atoms provide a unique vehicle for studying radiative processes over long times. In mid 1981, Kleppner (*Phys. Rev. Lett.* **47**, 233; 1981) proposed using such atoms to study the effects of cavity mode confinement in partially 'freezing out' spontaneous decay rates (*Nature* **293**, 514; 1981). A recent experiment by Vaidyanathan, Spencer and Kleppner (*Phys. Rev. Lett.* **47**, 1592; 1981) reports the observation of such waveguide effects, not on spontaneous transitions but on transitions induced in the Rydberg atoms by thermal blackbody radiation.

Rydberg atoms have been used before as detectors of far-IR and microwave blackbody radiation (*Nature* **279**, 476; 1979) but only in effectively unbounded space. Kleppner's new experiment reports a sudden increase in the induced lifetime of a

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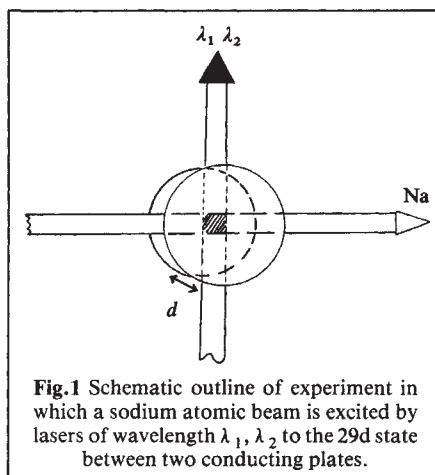


Fig.1 Schematic outline of experiment in which a sodium atomic beam is excited by lasers of wavelength λ_1, λ_2 to the 29d state between two conducting plates.

Rydberg atomic transition between two conducting plates when the resonant wavelength is increased beyond the waveguide cut-off (twice the plate separation d). The conducting plates are unable to support field modes of wavelength less than $2d$ in the direction normal to the plates. In addition, those modes parallel to the plates have their density altered but not turned off. Thermal radiation can only be supported on these modes so that transitions induced by blackbody radiation inside the plates are sensitive to the confining geometry.

The technique used to observe these waveguide effects by Kleppner and his

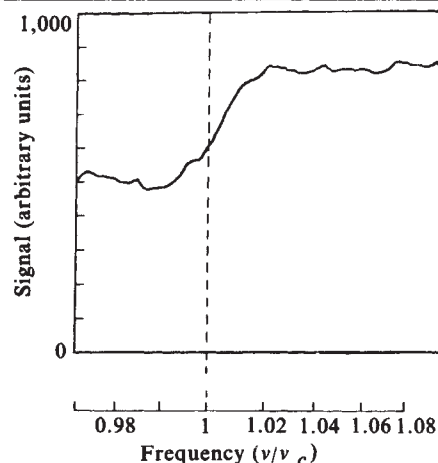


Fig.2 Blackbody radiative transfer signals 29d-30p in sodium between parallel conducting plates as the transition frequency ν is Stark tuned through the cut-off frequency $\nu_c = c/2d$ (from Vaidyanathan *et al.*).

group is particularly ingenious and is outlined schematically in Fig. 1. The sodium 29d Rydberg state is populated by stepwise absorption from two pulsed lasers of wavelengths λ_1 and λ_2 which drive the transitions 3s to 3p to 29d in an atomic beam travelling between two conducting plates separated by $1/3$ cm. The whole apparatus is placed in a shielded box at 180K so that the photon occupation number at wavelength $\lambda \sim 2d$ is approximately 86. All polarizations in the transition are possible. The transition 29d to 30p at a wavelength of $2/3$ cm ($\sim 2d$) is chosen for study and is excited by the thermal field at a rate of 300 per second. Transitions to other levels at $\lambda \neq 2d$ are of course also induced and contribute to a d -insensitive background.

The plate separation is kept fixed but the transition wavelength is varied by exploiting the large polarizability of Rydberg states to Stark-shift energy levels in a small d.c. voltage across the plates. The cut-off frequency $\nu_c = c/2d$ is 1.48 cm^{-1} . As the electric field across the plates is varied from 0.7 to 5.7 V cm^{-1} , the transition frequency varies from $0.97 \nu_c$ to $1.11 \nu_c$. The transition rate for the production of 30p atoms is measured by selectively ionizing only those 30p atoms making the transition, and counting the ions. As the transition frequency ν approaches the waveguide cutoff frequency ν_c , the rate of transition suddenly changes (Fig. 2): on the right-hand side, blackbody photons can fit between the plates, and on the left-hand side they cannot, decreasing the 30p production rate. As ν increases beyond ν_c , the modes normal to the plate surface are switched on. The Einstein relationships between stimulated and spontaneous transition rates imply that spontaneous decay was similarly affected in this experiment. The experiment of Vaidyanathan *et al.* is a beautiful example of how Rydberg atoms are sensitive to their immediate environment, making them the perfect testbed for what would otherwise be minute and unmeasurable effects. $\square$

ARTICLES

A seasonal signal in ocean currents to abyssal depths

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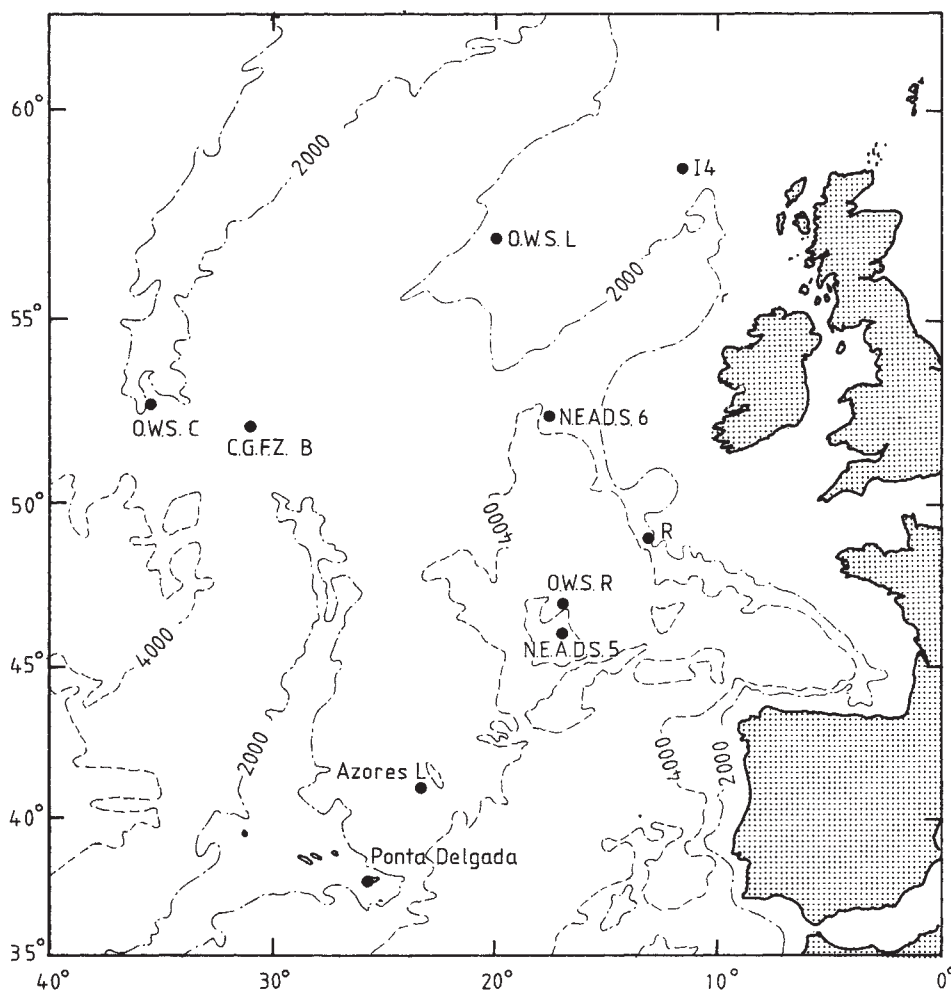
Long-term (9–32 month) current meter records from the eastern North Atlantic (41°N–59°N) suggest that, remote from the influence of the Western Boundary Current, eddy kinetic energy (K_E) at all depths is principally a function of wind stress and stratification, generated during the winter and early spring, propagating to abyssal depths and decaying to a minimum in late summer. Although this seasonal variation in K_E has not previously been described from the midlatitude ocean, some theoretical basis for it is presented.

In their analysis of long-term (644 days) current meter records from the northern Rockall Trough (IOS Mooring I4; 58°50' N 11°40' W; Fig. 1), Gould and Cutler¹ report a well-defined seasonal signal in eddy kinetic energy estimated at depths of 200, 1,000 and 1,500 m in 1,800 m water depth. Each record was first low-pass filtered using a gaussian filter similar to that described by Schmitz² with a high frequency cutoff around 0.5 cycles per day (c.p.d.). Finding clear evidence of energetic winter fluctuations in the filtered time series, see for example,

Fig. 2a, each record was then divided into 60-day pieces each overlapping by half their length, and for each piece the eddy kinetic energy (K_E) over a selected band of periods (2.92–40 days) was estimated using spectral analysis techniques similar to those defined by Schmitz and Hogg³. From this the seasonal nature of the fluctuations is obvious (Fig. 2b).

We extend this analysis to a widely-scattered range of sites in the eastern North Atlantic and to water depths > 4,700 m using long-term (9–32 month) records obtained by the Ministry of

Fig. 1 Location of six current-meter moorings and three Ocean Weather Stations. The 2,000-m and 4,000-m isobaths are also shown.



Agriculture, Fisheries and Food (MAFF), Fisheries Laboratory, Lowestoft and the Institute of Oceanographic Sciences (IOS), Wormley. The 60-day piece-length and low-pass filtering techniques are standard throughout; however, the period bands used vary, to accommodate records of different spectral characteristics and because of differences in the spectral analysis procedures at the two laboratories. By the Blackman-Tukey method⁴ (MAFF) spectral estimates are derived for frequencies which are fractions of the number of lags ($L = 20$ throughout) while the FFT method⁵ (IOS) obtains estimates at frequencies corresponding to fractions of the length of record ($t = 60$ days). Thus generally,

$$K_E(\nu_1\nu_2) = \Delta \sum_{\nu_1}^{\nu_2} K_E(\nu)$$

where $K_E(\nu_1\nu_2)$ is the fluctuation (eddy) kinetic energy between frequencies ν_1 and ν_2 , and Δ is the bandwidth of the spectrum ($1/2L$ or $1/t$ according to method). In practice, K_E estimates are reported below for two bands of periods, $K_E(3-27)$ covering periods from 3 to 26.7 days and $K_E(3-80)$ covering those from 3 to 80 days. Where the earlier results of Gould and Cutler are described, $K_E(3-40)$ denotes periods from 2.92 to 40 days. $K_E(\text{Rec})$ denotes the total kinetic energy per unit mass associated with the entire range of 'eddy' frequencies accessible in the full low-passed record ($\nu = 1/2t$ to $1/2$ c.p.d.), that is $K_E(\text{Rec}) = 1/2[\text{var } u + \text{var } v]$, where $\text{var } u$ and $\text{var } v$ are the variances of the east and north velocity components respectively.

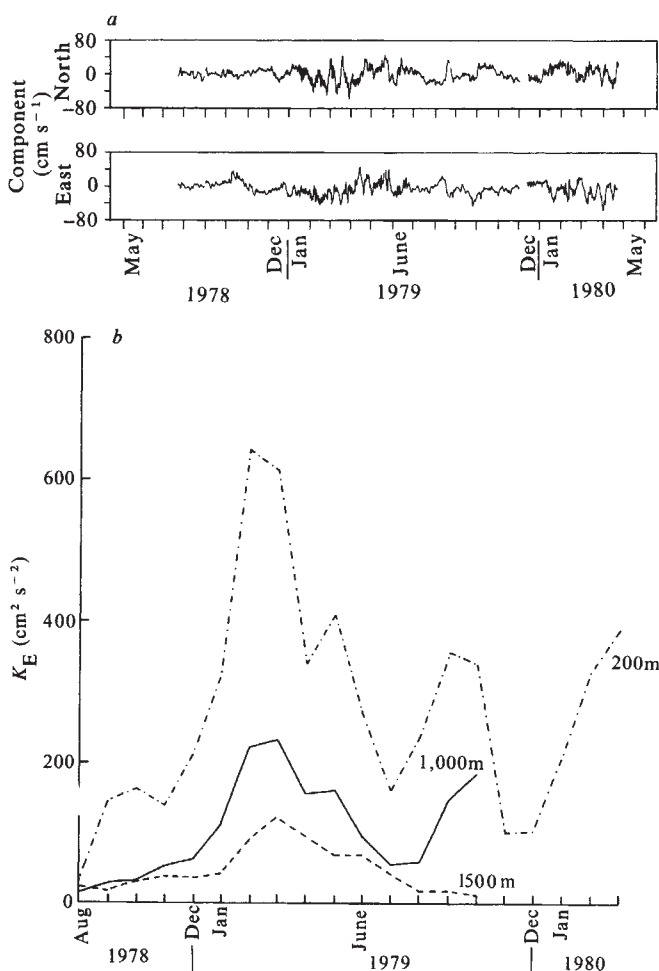


Fig. 2 *a*, Low-pass filtered time-series of north and east current components at Mooring I4 (Rockall Trough; 58°50' N 11°40' W), 200 m depth, July 1978–April 1980. *b*, Estimates of eddy kinetic energy in the period band 3–40 days [$K_E(3-40)$] at 200-, 1,000- and 1,500-m depth, Mooring I4 (Rockall Trough). Individual estimates are for 60-day pieces of record, each overlapping by half their length.

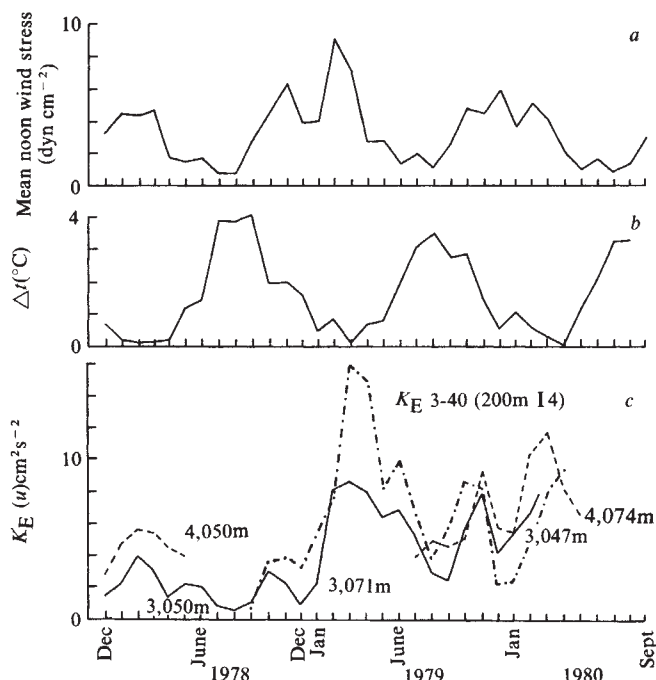


Fig. 3 *a*, Mean noon wind stress at OWS LIMA [57° N 20° W], December 1978–September 1980. *b*, 0–450 m temperature difference at OWS LIMA, from XBT observations. *c*, Time-variation of eddy kinetic energy [$K_E(3-27)$] in the east-west current component at 3,050-m (—) and 4,050 m-depth (---), NEADS-6 mooring [52°30' N 17°45' W]. The 200-m curve of $K_E(3-40)$ from Mooring I4 (Rockall Trough) has been superimposed with a 1-month lag (····) to show the close correlation and the apparent 1-month delay in the deep water.

Seasonal signal in eddy kinetic energy

The records from the Rockall Trough (Fig. 2*b*) appeared to show three characteristics, apart from the obvious seasonality of $K_E(3-40)$. First, the amplitude of the seasonal peak is markedly depth dependent, varying from ~ 640 cm² s⁻² at 200 m to ~ 120 cm² s⁻² at 1,500 m. Second, the curves seem to lag in time with increasing depth; from 200 m to 1,500 m for example the primary winter peak is delayed by ~ 1 month. Third, there are signs that the main winter peak centred on February–March is preceded by a secondary peak of smaller amplitude in September–October at the time of the equinoctial gales (but before the complete eradication of the seasonal thermocline).

Figure 3 extends this analysis to greater depths using the multiyear records from the NEADS-6 site [52°30' N 17°45' W; North-east Atlantic Dynamics Study] on the continental rise at the mouth of the Rockall Trough (see Fig. 1). This mooring has been operated continuously by MAFF since October 1977. From 60 day overlapping pieces of the low-passed records, Fig. 3*c* illustrates the time-variation of eddy kinetic energy [$K_E(3-27)$] in the east-west (along isobath) current component for the 830 day record at 3,050 m and for the discontinuous 602 day record at 4,050 m. Water depth is $\sim 4,100$ m. On the same time-base, Fig. 3*a* describes the mean noon wind stress for each month at OWS LIMA [57° N 20° W] calculated using a quadratic wind stress law with variable drag coefficient as defined by Heaps⁶, and Fig. 3*b* shows the 0–450 m temperature difference at LIMA from XBT observations.

Figure 3 confirms many of the features found in the Rockall Trough, though in this case at abyssal depths: roughly coincident with the winter windstress peak and with the eradication of the seasonal thermocline, there is evidence for a February–March peak in the u contribution to $K_E(3-27)$ in each of the 3 years, preceded by a lesser peak in October–November (at the time of the equinoctial gales but when some residual stratification remains in the upper water column).

Figure 3 also gives some indication as to the scale of the forcing and on the phase lag with depth. In Fig. 3c the 200 m curve of $K_E(3-40)$ at I4 (Rockall Trough) has been superimposed on the NEADS-6 time series with a 1-month lag to show both the close correlation and the apparent 1 month delay in the deep water. Despite a horizontal separation of >800 km between I4 and NEADS-6, the correlation is sufficiently close between the 200 m (I4) and 3,050 m (NEADS-6) records at 1-month lag to suggest a common broad-scale forcing (over the 19-month overlap period $r=0.76$ though with independent estimates only every 2 months). Note that although the I4 mooring had its main buoyancy close to the surface (150 m), the tidal constituents of the records are constant from season to season, suggesting that the records are free from wave-induced contamination. In the case of the NEADS-6 mooring there is no possibility that the observed variation in 'eddy' energy could be due to wave action or to mooring motion arising from it as the uppermost part of the mooring lay at 3,000 m depth throughout the 32-month period of record. (The same is true for all other MAFF moorings described below, whose main subsurface buoyancy varied from 1,000 to 4,000 m depth.) Depth-modified Aanderaa RCM-4 instruments were used throughout.

The 3,050 m time-series in Fig. 3c was used to investigate the spectral composition of the additional 'winter' eddy kinetic energy. The 343 day series from the second deployment at this depth was halved to provide two records of 172 day representing the quiescent 'summer' half year (1 July 1978–19 December 1978) and the energetic 'winter' half year (19 December 1978–8 June 1979). Figure 4a shows the winter minus summer spectral energy density for both the u and v spectra. At the lowest and highest frequencies of the spectrum there is little difference in energy between summer and winter, but each component shows a sharp winter excess of energy at (different) intermediate frequencies. The winter excess in the u (along isobath) component is of greater amplitude and peaks at a longer period (25 days) than the v component (12.5 days) in this particular pair of half-years. It is also the more conservative in time. Of the three possible (overlapping) adjacent pairs of winter–summer periods in the 3,050 m record, and in the single (discontinuous) winter–summer pairing of the 4,050 m record, the winter excess of eddy energy peaked at 33, 25, 25 and 25 days respectively in the u component while the corresponding peaks in the v component were much more variable and ill-defined (except for the pairing shown in Fig. 4a).

Figure 5 examines records from four additional sites between January 1977 and May 1980 to test whether the seasonal signal in eddy energy is restricted merely to the Rockall area or is a more widespread and general characteristic of the eastern North Atlantic. In most cases, estimates of $K_E(3-27)$ and $K_E(3-80)$ are superimposed. Locations of the moorings and of the three Ocean Weather Stations discussed are shown in Fig. 1.

Figure 5 a–e displays results from the NEADS-5 site at $\sim 46^\circ$ N 17° W, in the foothills of the Mid Atlantic Ridge close to its junction with the Porcupine Abyssal Plain. Figure 5a, b shows IOS records (452 and 425 days respectively) from 600 and 3,000 m depth, while Fig. 5c–e (the deepest records discussed here) is from a later 1-yr reoccupation of the site by MAFF. Of Fig. 5f–i (all MAFF) Fig. 5f, g shows 253 day records at 2,977 and 3,977 m depth from the lower north wall of the Charlie Gibbs Fracture Zone, Southern Trench, at $52^\circ 09'$ N $31^\circ 00'$ W⁸, Fig. 5h is a 340 day record from 4,046 m depth on the hilly eastern flanks of the Mid Atlantic Ridge, north-east of the Azores [$41^\circ 00'$ N $23^\circ 18'$ W] and Fig. 5i is a 1-yr record at 2,049 m depth from the European Continental Slope ($48^\circ 59'$ N $12^\circ 53'$ W).

Coupling these results with the earlier discussion of I4 and NEADS-6 we find:

(1) At all six sites, ranging in latitude from 41° N to 59° N, ranging in sampling depth from 200 to 4,710 m and with records ranging in total eddy kinetic energy [$K_E(\text{Rec})$] from the highest values yet encountered in the eastern basin [$K_E(\text{Rec}) = 196 \text{ cm}^2 \text{ s}^{-2}$ at 200 m, I4] to very nearly the lowest [$K_E(\text{Rec}) =$

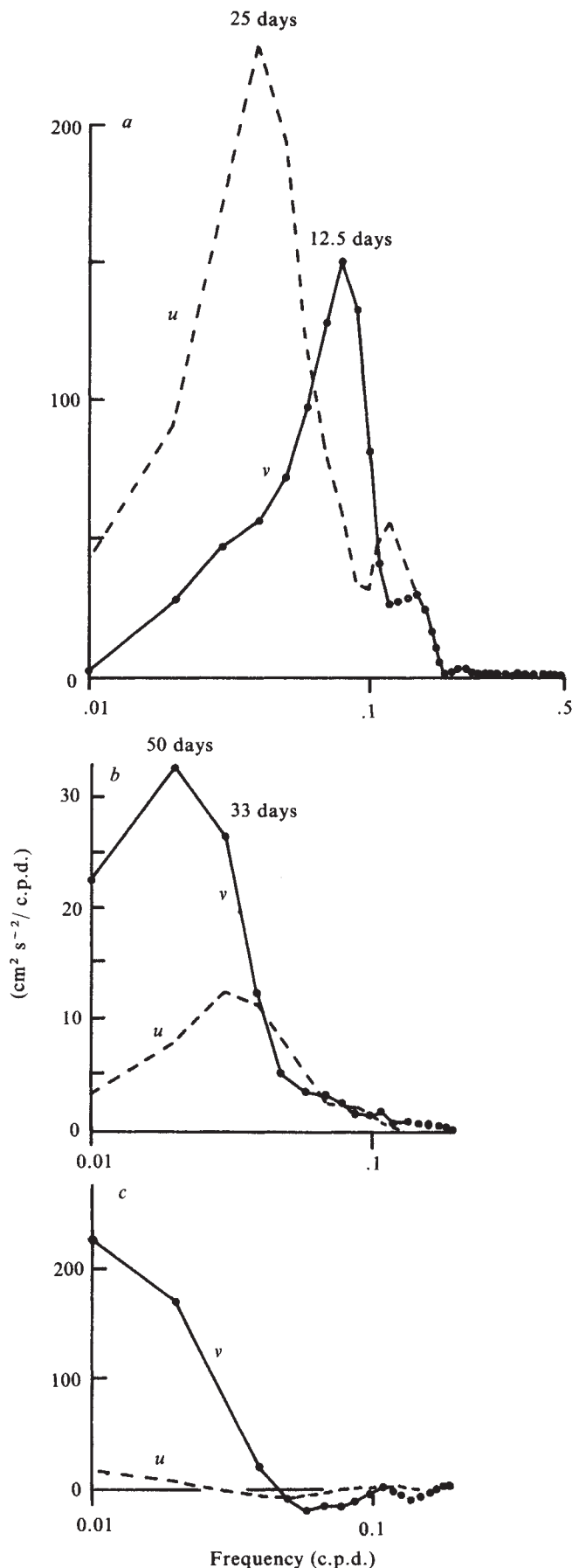


Fig. 4 Winter minus summer spectral energy density for both the zonal (u) and meridional (v) current components at: a, 3,050-m depth, NEADS-6 ($52^\circ 30'$ N $17^\circ 45'$ W); b, 4,050 m depth, NEADS-5 (46° N 17° W); c, 2,049-m depth, mooring R ($48^\circ 59'$ N, $12^\circ 53'$ W; European continental slope).

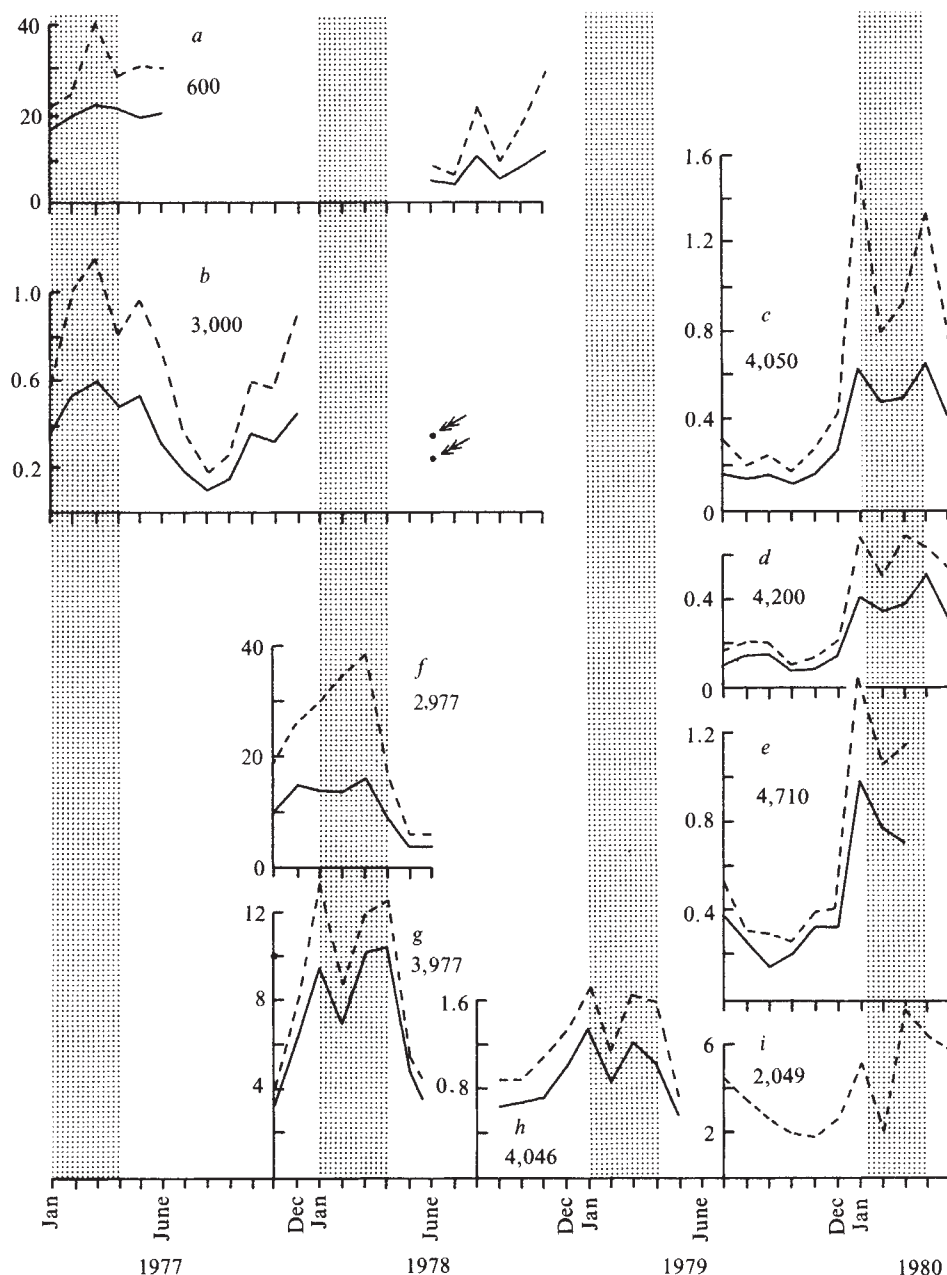


Fig. 5 Time series of eddy kinetic energy estimates in the period bands 3–27 days (—) and 3–80 days (---) at *a*, 600-m depth, NEADS-5 [46° N 17° W]; *b*, 3,000-m depth, NEADS-5; *c*, 4,050-m depth, NEADS-5; *d*, 4,200-m depth, NEADS-5; *e*, 4,710-m depth, NEADS-5; *f*, 2,977-m depth, mooring B, Charlie-Gibbs Fracture Zone, southern trench [52°09' N 31°00' W]; *g*, 3,977-m depth, mooring B, Charlie-Gibbs Fracture Zone, southern trench; *h*, 4,046-m depth, mooring L, Azores Array [41°00' N 23°18' W]; *i*, 2,049-m depth, mooring R, European continental slope [48°59' N 12°53' W].

0.6 at 4,200 m, NEADS-5 (ref. 9)], there is a clearly-defined seasonal variation in $K_E(3-27)$ or $K_E(3-80)$.

(2) All these sites are characterized by some degree of bottom slope, with either gently-sloping, hilly or abrupt topography. Conversely (though not shown here), no clear seasonal variation in K_E could be detected at sites on the flat abyssal plains, for example, at MAFF sites N, O or P on the Porcupine Abyssal Plain⁹ (within the NEADS-6–NEADS-5–R triangle) or at the NEADS-3 site on the Iberian Abyssal Plain (42° N 14° W).

(3) The dominant period of the 'excess' winter energy has been examined for four records only (two at NEADS-6) but seems to be highly variable between sites. The 25-day and 12.5-day dominant peaks of excess winter energy in the u and v spectra at NEADS-6 (3,050 m) have already been described (Fig. 4a). At 4,050-m depth on the NEADS-5 site the corresponding peaks are at 33 days and 50 days (Fig. 4b) while at 2,049 m at mooring 'R' the excess winter energy is still increasing at the longest periods of the spectrum (≥ 100 days; Fig. 4c).

(4) In each case the dominant winter excess of energy occurs in the along-isobath component (u for NEADS-6; v for NEADS-5 and 'R') and although any association with topography may be coincidental the dominance of one component over the other appears least on the gentle slope of the continental rise (Fig. 4a)

and greatest on the steep continental slope. In the latter case the winter excess of energy is almost solely in the along-slope component (Fig. 4c).

(5) The apparent ~1-month phase-lag between the time variations of K_E in the upper layer and the deep water is supported in Fig. 5, provided that the supposed link with wind stress is a valid (causal) one. During July 1979–May 1980 deep current measurements to 4,710-m depth at NEADS-5 can be matched with wind stress at the neighbouring OWS ROMEO site (see Fig. 1). At OWS ROMEO the winter wind stress peaked sharply in December 1979, 1 month before the K_E at depth rose to its winter maximum (Fig. 5c–e).

(6) The supposed link between eddy kinetic energy and wind stress is given some credence by the general 'shape' of the curves in Fig. 5. At all sites, depths and sampling periods shown, the time variation of K_E is surprisingly similar; following a broad summer minimum centred on September, the subsequent winter peak from January to April is characteristically bimodal, interrupted by a secondary minimum in February. Curves of monthly mean noon windstress for the relevant years at OWS CHARLIE, LIMA and ROMEO and at Ponta Delgada, Azores (Fig. 6) show that this midwinter 'dip' is also a characteristic feature of the windstress field in January/February.

(7) To obtain some estimate of the seasonal contribution to the total kinetic energy of the long-term record, 1-yr records from the second deployment at NEADS-6 (3,050 m) and from 4,050 m at NEADS-5 were split into equal ~6-month periods representative of the quiescent summer and energetic winter periods. The total eddy kinetic energy per unit mass of the summer, winter and full-record time series was then calculated. At 3,050 m, NEADS-6, $K_E(\text{Rec})_{\text{summer}} = 3.72 \text{ cm}^2 \text{ s}^{-2}$, $K_E(\text{Rec})_{\text{winter}} = 15.64 \text{ cm}^2 \text{ s}^{-2}$ and $K_E(\text{Rec})$ for the full record = $9.72 \text{ cm}^2 \text{ s}^{-2}$; for 4,050 m, NEADS-5, the corresponding figures are 0.48, 1.39 and $1.02 \text{ cm}^2 \text{ s}^{-2}$ respectively. Thus the summer:winter eddy energy is in the ratio of 1:4.2 for the NEADS-6 record and 1:2.9 for that at NEADS-5. The corresponding ratios of summer:winter mean wind stress for 6-month periods, 1 month in advance of the current meter data, are 1:2.2 (LIMA) and 1:1.9 (ROMEO).

Theoretical mechanism

From reviews^{10,11} of the known properties of linear wave propagation in a rotating stratified ocean we can identify possible driving forces for the seasonally-varying low-frequency oscillations that we have observed. Candidate mechanisms include: (1) a response to local and/or remote forcing at the surface by wind and buoyancy effects; (2) instabilities and/or eddy-shedding over topography in a seasonally-varying mean flow; (3) instabilities of large-scale wave motions (such as planetary waves) driven by surface forcing; (4) propagation from equatorial regions of coastal or Kelvin waves on the eastern boundary which then propagate to the interior either directly as planetary waves or indirectly by following depth (h) or dynamical (f/h) contours as topographic or edge waves ($f = 2\Omega \sin \phi$, where Ω is the Earth's angular rate of rotation and ϕ is the latitude); (5) wave trapping by closed (f/h) contours;

Many of these theoretical mechanisms have been observed in the ocean but mechanism (1) appears to be the most relevant in view of the close association with wind stress and stratification in our results. This mechanism was explored using an idealized model¹² which includes a crude representation of stratification, rotation, topography, coastal boundaries and surface wind forcing.

It is important to decide whether the observed waves are likely to be forced or free. The maximum frequency (ω_{max}) of a free planetary wave is

$$\omega_{\text{max}} = -\frac{1}{2}\beta a$$

where β is the northward gradient of the Coriolis parameter and a is the local radius of deformation (that is the distance over which gravitational and rotational forces are balanced). Using

baroclinic/barotropic to refer to depth dependent/independent behaviour, we find that for barotropic modes a is ~2,000 km at midlatitudes so that ω_{max} corresponds to a period of 3.6 days. Thus barotropic waves could contribute to the observed oscillations but have no vertical dependence and so cannot account for the observed vertical structure. For a first baroclinic mode a is ~30 km and ω_{max} corresponds to a minimum period of ~8 months. Thus the oscillations observed at 12–50-day periods cannot be free baroclinic planetary waves (similar calculations for other wave modes are more difficult to make without additional data). Therefore it was assumed that the observed oscillations are not free and we now discuss the forced response to wind. In the present model, the ocean is considered to have two layers of mean depth H_1 and H_2 ($H_1 \ll H_2$) with mean densities ρ_1 and ρ_2 ($\rho_1 < \rho_2$). The stratification is stable but unvarying. Rigid boundaries run north-south but none east-west. Any topography (of height T) is shallower than the lower layer thickness and does not vary north-south. The ocean is driven by an eastward windstress acting on the upper layer such that the response is periodic north-south. A full description of the model is given elsewhere¹².

In view of the depth-dependence of the observed oscillations our attention is focused on the equation in ref. 12 describing the baroclinic component of zonal flow. Initial runs using realistic input parameters but flat topography confirm that for the seasonal cycle the planetary wave can propagate, with a rapidly oscillating solution across the basin, but at the (lower) 'eddy' frequencies observed no propagation is possible and $|\hat{u}|$ (the magnitude of the zonal baroclinic component) is small away from the boundaries. The solution is almost purely barotropic as anticipated.

To see if topography orientated north-south can produce a distinct baroclinic signal, the calculation was repeated with topography varying sinusoidally east-west, with amplitude 1,500 m (in an ocean of depth 5,400 m) and wavelength 1,250 km. The results in ref. 12 show that in these circumstances the topographic effect must be unrealistically large to overcome the time-dependence effect and yield a sizeable baroclinic signal. The ratio of the topographic to time-dependent terms is dimensionally $g'Tx l(H_1 H_2^{-1})^2 \omega^{-1} f^{-1}$, where g' is the reduced gravity, $[g(\rho_2 - \rho_1)\rho_2^{-1}]$, Tx the topographic slope, and l the north-south wave number of the forcing. For the above parameters, this ratio is about 5×10^{-3} . However, the ratio can easily be increased, by steeper topography, a shorter north-south length scale, or by increasing H_1 . All three of these effects are probably relevant in the eastern North Atlantic: the topographical variations can be quite rapid; the confused topography may well induce shorter north-south scales in the oceanic response; and, most encouragingly, the increase in eddy kinetic energy at depth does not occur until a seasonally-strengthening wind stress is accompanied by the breakdown of the seasonal thermocline (that is a rapid increase in H_1). Thus preliminary results from this simple model seem capable of reproducing a baroclinic signal at depth and at the observed eddy frequencies.

Discussion

There are many potential sources of error in the above type of analysis. The spectral analysis techniques are strictly valid only for stationary time series which is not the case in the 60-day pieces of record that were used. The current meter records themselves are relatively short, sparse or discontinuous and rarely provide concurrent full-depth coverage. On the link with wind stress, there is an obvious need for caution in exploring causality between time-series that are dominated by one narrow-band signal such as the seasonal cycle. With one exception, moorings are remote from the Ocean Weather Stations which provide the wind stress data, and although the degree of thermal stratification is thought to be important to the input of eddy energy to the deep ocean, time series of temperature in the upper ocean which define the breakdown and re-establishment of the stratification are very rare.

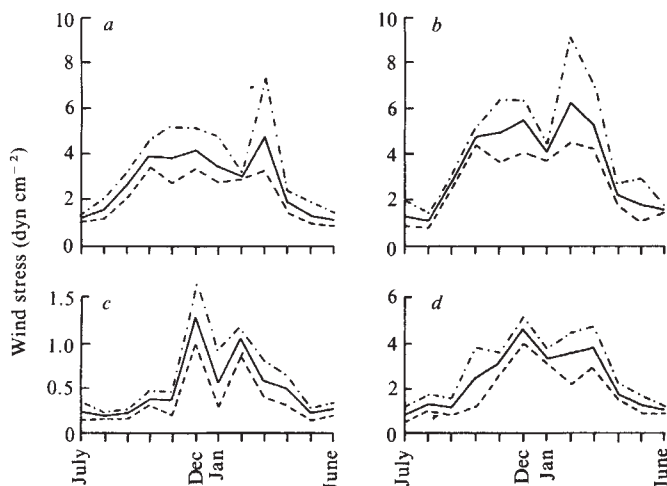


Fig. 6 Maximum, minimum and mean noon wind stress for each calendar month at *a*, OWS CHARLIE, 52°45' N 35°30' W (1977–79); *b*, OWS LIMA, 57° N 20° W (1978–80); *c*, Ponta Delgada, Azores (1977–79); *d*, OWS ROMEO, 47° N 17° W (1977–79).

Nevertheless, the seasonal signal in fluctuation kinetic energy is plainly visible in time series which have been subjected to no more sophisticated processing than the application of a low-pass filter (for example, Fig. 2a), and where spectral analysis has been used, the seasonal signal in K_E is so consistently shown over a wide range of sites, years and sampling depths as to suggest that it is both real and widespread, not merely an artefact of the analysis. We conclude therefore that in the eastern North Atlantic, remote from the influence of the Western Boundary Current, eddy kinetic energy at all depths is principally some function of wind stress and stratification, generated during the winter and early spring, propagating to abyssal depths and decaying to a minimum in late summer. If so a similar seasonal signal will probably be found to dominate other records from the eastern midlatitude oceans and may even be detectable within the fields of influence of the western boundary currents or further afield in the equatorial zone. (A significant correlation between wind stress and deep ocean currents has recently been observed in the Atlantic North Equatorial Current, east of Barbados, at week to month time scales¹³). Note that the suggested mechanism seems to require some degree of bottom

slope, a point which is apparently confirmed in the observations.

The above conclusion is not meant to imply that mesoscale eddies as conventionally understood do not exist in the north-east Atlantic. Plainly they do, as demonstrated by much direct and indirect evidence¹⁴⁻¹⁶, but equally these random and presumably non-seasonal features are sufficiently sparse (or weak or shallow) to be undetectable against the dominant 'eddy' signal arising from wind stress. To paraphrase the conclusion of Bernstein and White for the north-east Pacific¹⁷, the north-east Atlantic appears to be a 'near void' for mesoscale eddies. This is a rather different conclusion to that of Wyrski *et al.*¹⁸ who suggest, from their analysis of ship drift data "that the eddy motion in the ocean is generated in areas of strong mean shear flow and is subsequently distributed over the whole ocean with much less decay than the mean flow". The difference is important, however. If the eddy climate of the eastern midlatitude ocean really is more properly described as a function of windstress and stratification than as the synthesis of random mesoscale events, then the problem of its parameterization in general circulation models should be simplified.

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Hydrothermal petroleum in mineralized mounds at the seabed of Guaymas Basin

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Petroleum has been dredged from an active hydrothermal mound area in the southern rift of Guaymas Basin, Gulf of California. This organic matter is composed of gasoline-range aliphatic and aromatic hydrocarbons and predominantly residual polar asphaltic material. The aliphatic hydrocarbons of two bitumen samples have very different boiling range and composition. Both samples contain polynuclear aromatic hydrocarbons and olefins, which indicate formation at pyrolytic temperatures. The overall compositional data indicate an origin from biological detritus by thermal alteration and rapid quenching by hydrothermal removal, followed by condensation at the seabed.

THE Guaymas Basin is an actively spreading oceanic basin, part of the system of spreading axes and transform faults that extend from the East Pacific Rise to the San Andreas fault system^{1,2}. The basin is tectonically very active and consists of two rift valleys, the northern and southern troughs, separated by a 20-km transform fault area. The sediment blanket accumulates at a high rate ($>1 \text{ m kyr}^{-1}$), keeping the basin floor covered, while the ocean plate accretion process occurs by dyke and sill intrusions into the unconsolidated muds^{1,3}. Both rifts have high conductive heat flow (locally exceeding 1.2 W m^{-2})^{4,5}.

Petroliferous gasoline-range hydrocarbons were reported in a gravity core from the northern trough and interpreted to be of a thermogenic origin⁶. Interstitial gas, bitumen and kerogen analyses of samples from Deep Sea Drilling Project Sites 477 and 481 confirm the alteration of the indigenous organic matter by hydrothermal stress^{3,7-10}.

The samples discussed here are derived from a deep-tow plus heatflow survey cruise by Scripps Institution of Oceanography in 1980. Numerous hydrothermal mounds rising 20-30 m above the rift floor (water depth ~2,000 m) were mapped in the southern trough (Fig. 1) and some had active hydrothermal plumes¹¹. Photographs showed encrusted mounds and columns covered with tubeworms and other fauna. A dredge haul (7D, Fig. 1) across a 50 m-wide patch of sinter deposits which form a mound recovered claystones, massive sulphides, barite, talc and other hydrothermal minerals, together with tubeworm specimens. Many of the fragments in the dredge were stained with a petroleum-like oil and had a strong odour similar to diesel fuel.

Several hand-specimens of these odorous, oil-stained samples were sealed in foil and glass jars, then analysed some months later to determine the nature, composition and source of the petroliferous material. One specimen (7D-2B) is a massive sulphide with approximately equal proportions of sphalerite, chalcopryrite and pyrrhotite, and interstitial barite; the others are claystones.

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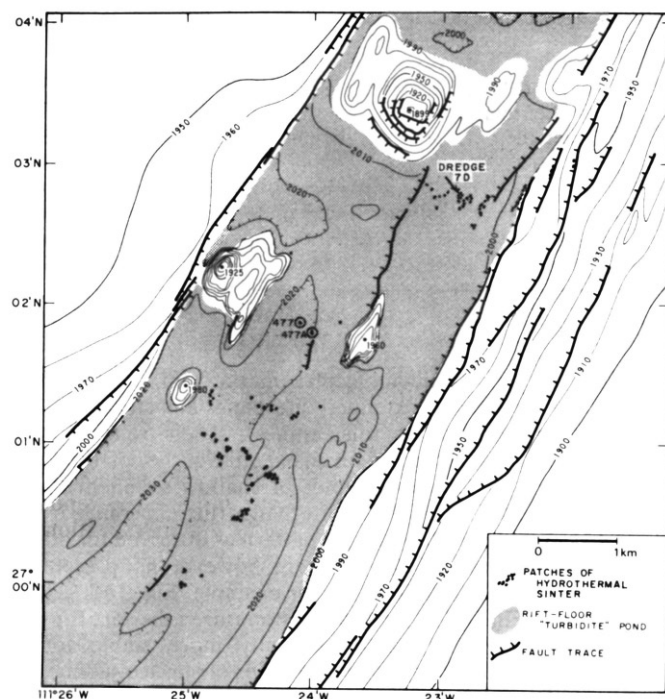


Fig. 1 Location map showing the dredge site (7D) and DSDP Site 477 in the southern rift of Guaymas Basin.

Experimental

Two of the dredge sub-samples (7D-3B and -5A; Fig. 1) were canned under water for analysis of the gasoline-range hydrocarbons as described⁶. A packed column (stainless-steel, 2 m × 2 mm i.d., 0.3% Carbowax 20M on Carbopac C) was used for the analytical gas chromatography (Hewlett-Packard Model 5830A). The injector temperature was at 200 °C and the temperature programme was: 30 °C (1 min), 30–225 °C (5 min). The headspace in the cans was 150 cm³ and the concentrations of the hydrocarbons are reported as ng cm⁻³ of headspace.

Two other dredge sub-samples (7D-2B and -4A, B; Fig. 1) were first extracted as received with methanol and methylene chloride (CH₂Cl₂). The aqueous phase was re-extracted with CH₂Cl₂ and the extracts were combined. The residue was subsequently crushed and extracted further with CH₂Cl₂, which was also combined with the initial extract. The total extracts were concentrated and aliquots were subjected to column chromatography on silica gel (column 1 × 17.5 cm). The samples were separated by elution with hexane (15 ml as F1), hexane/benzene (3:2, 25 ml as F2) and then methanol (20 ml as F3) into the respective fractions, which were analysed by capillary gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The total extracts were also quantified by weighing aliquots of dried extract (or fraction) on a microbalance.

The capillary GC analyses were carried out on a Hewlett-Packard Model 5830 GC with a fused silica capillary column (25 m × 0.20 mm i.d.) wall-coated with SP-2100. The operating conditions were as reported earlier⁶. The GC-MS analyses were conducted on a Finnigan Model 4000 quadrupole mass spectrometer interfaced directly with a Finnigan Model 9620 GC and equipped with a 30 m × 0.25 mm i.d. fused silica capillary column (wall-coated with SE-54). The operating conditions were as reported earlier⁶. The MS data were acquired and processed with a Finnigan-Incos Model 2300 data system. Compound assignments were made from individual mass spectra and GC retention times, with comparison to authentic standards where possible.

Results and discussion

The relative amounts of the major gasoline-range hydrocarbons of two dredge samples are listed in Table 1. Sample 7D-5A has a

much greater amount of C₅–C₁₀ hydrocarbons than sample 7D-3B and in both cases the concentrations of branched and cyclic compounds (possibly also olefins) far exceed the abundance of the normal hydrocarbons. The approximate total concentration of C₂–C₁₀ hydrocarbons for sample 7D-5A is about 10 times greater than for the other sample. The relative distributions of all hydrocarbons are very similar for both samples in the range of C₅–C₁₀, but sample 7D-3B has only minor components from C₂ to C₅ (Table 1). The large amounts of C₂–C₁₀ hydrocarbons and their structural diversity confirm their origin by thermal generation from the sedimentary organic matter⁷.

The total bitumen (lipid) extract of sample 7D-2B is light amber in colour and black for sample 7D-4A, B; both extracts of the samples exhibit blue fluorescence in solution (Table 2). This colour is typical for (naphtha) heavy condensates and the concentrated extract for sample 7D-4A, B does exhibit a yellow fluorescence, indicating a major amount of heavier oil components. The total yield of bitumen is highest for sample 7D-4A, B and it contains only traces of elemental or organic sulphur. The 'light' bitumen (7D-2B), however, has a major proportion of elemental sulphur and also organic sulphur compounds (the minerals of this sample are massive sulphides). These two bitumens are clearly different, which is further substantiated by the following data. The hydrocarbon yields are found in Table 2, where the aliphatic fraction (F1) is lowest for both samples and most of the bitumen is comprised of asphaltic (F3) and then aromatic/naphthenic (F2) material. The aromatic-to-aliphatic ratio for sample 7D-2B is 9.3 and it is 4.7 for sample 7D-4A, B, typical for many crude oils¹².

The GC traces of the aliphatic hydrocarbons are given in Fig. 2 and they are dramatically different. Sample 7D-2B (Fig. 2a) exhibits a pattern typical of a petroleum, where the dominant *n*-alkanes, C_nH_{2n+2}, range from *n* = 12 to 33, with no carbon number predominance (CPI_{12–33} = 1.03) and a maximum at *n*-C₂₁. Carbon preference index (CPI) = odd carbon number *n*-alkanes/even over the range indicated for example,

$$\text{CPI}_H = \frac{2\sum \text{odd } C_{13}-C_{33}}{\sum \text{even } C_{12}-C_{30} + \sum \text{even } C_{14}-C_{32}}$$

Pristane and phytane are about equal (Pr/Ph = 1.06). The complex, unresolved mixture of branched and cyclic hydrocarbons (that is, hump) ranges from C₁₁ to C₃₁, also typical of petroleum. On the other hand, sample 7D-4A, B (Fig. 2b) exhibits a much narrower GC profile, skewed to lower carbon

Table 1 Volatile hydrocarbons in dredge samples from Guaymas Basin

Compound*	Retention time (min)	Approximate concentration (ng cm ⁻³ of headspace—150 cm ³ total)	
		7D-3B	7D-5A
2-Methylbutane	5.1	2.6	14
<i>n</i> -pentane	5.4	1.4	39
Cyclohexane	7.3	0.5	6
4-Methyl-2-pentene	8.0	0.8	6.3
3-Methylpentane	8.3	1.4	11
2-Methylpentane + benzene	8.6	0.9	44
<i>n</i> -Hexane	9.7	9.2	260
C ₂ -Cyclopentane	10.3	3.3	46
Methylcyclohexane	10.7	14	1,050
3-Methylhexane	12.1	3.3	—
2-Methylhexane	12.4	5.8	210
<i>n</i> -Heptane + toluene	13.5	>130	950
<i>n</i> -Octane	16.4	24	29
<i>n</i> -Nonane	19.2	48	26
<i>n</i> -Decane	22.2	17	40
Total C ₂ –C ₁₀ hydrocarbons (μg)		80	875

* Identifications based on GC retention time only.

Table 2 Yields and composition of bitumen from dredge samples in Guaymas Basin

Sample	Colour	Total bitumen extract		Sulphur ($\mu\text{g S}^\circ$ per g)	Aliphatic HC (F1)		Aromatic/naphthenic hydrocarbons (F2)		Asphaltic (NSO) compounds (F3)	
		Fluorescence (UV)	Total yield (mg) ($\mu\text{g per g}$)		(μg per g)	(% of total bitumen)	(μg per g)	(% of total bitumen)	(μg per g)	(% of total bitumen)
7D-2B	Light amber	Sky blue	110 1,075	~450	27	2.5	250	23.2	315	29.3
7D-4A, B	Black	Dark blue (yellow on sample matrix)	8,730 70,400	Traces	4,850	6.9	22,700	32.0	42,900	61.0

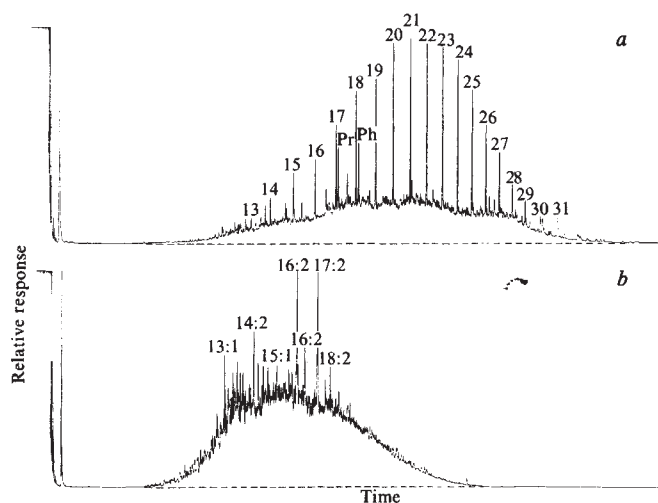


Fig. 2 Gas chromatograms of the aliphatic hydrocarbon fractions (F1) for: *a*, sample 7D-2B; and *b*, sample 7D-4A, B. The carbon chain length of the *n*-alkanes is indicated by the arabic numerals, olefins are indicated by chain length: double bond equivalent, Pr = pristane, Ph = phytane. GC conditions as cited in the text.

numbers. The dominant resolved peaks are various mono- and di-olefins ranging from C_{12} to C_{19} and the hump ranges from C_9 to about C_{21} . This is very unlike typical petroleum, as olefins are not found in mature crudes^{12,13}. Olefinic hydrocarbons have been identified in a crude oil from Bradford, Pennsylvania¹⁴ and they were found to consist of mid-chain normal, branched and cyclic analogues with the double bonds mainly in the *trans* configuration. The presence of these olefins was accounted for by a process of natural thermal cracking followed by the rapid disappearance of *cis* and terminal olefins¹⁴. The *n*-alkanes of sample 7D-4A, B are present as minor components and range from C_{10} to C_{23} , with essentially no carbon number predominance ($CPI_{10-23} = 1.20$) and a maximum at C_{15} . Pristane is more abundant than phytane (Pr/Ph = 1.6).

The GC-MS data for sample 7D-2B (F1) confirmed the minor presence of a similar range of mono- and di-olefins ranging from C_{12} to C_{19} . These compounds are not terminal olefins as were identified near the sills of DSDP site 481A (ref. 9). They are slightly more stable 'in-chain' olefins with methyl branching, similar to those described by Hoering¹⁴.

The major diagnostic molecular markers in this fraction consist of triterpenoids, extended diterpanes and steranes with their rearranged analogs (diasteranes). Their relative distributions are given in Fig. 3 with an example of the corresponding compounds in unaltered lipids of shallow sediment from Guaymas Basin⁶. The extended diterpanes (Fig. 3*a*) range from C_{20} to C_{29} in a similar distribution pattern as observed for other mature petroleum samples¹⁵. They are, however, not present in the unaltered surface sediments (for example, Fig. 3*b*)^{6,9}. The triterpenoids (Fig. 3*a*) are surprisingly 'mature'; they are for the most part in their thermodynamically more stable form, completely different from unaltered lipids of surface sediments (for example, Fig. 3*b*). The triterpenoids are comprised primarily of the $17\alpha(H)$, $21\beta(H)$ -hopane series ranging from C_{27} (no C_{28}) to C_{35} and the homologues from C_{31} to C_{35} are present as 22-S and R diastereomeric pairs in a ratio of about unity. This series constitutes the stable mature form of these compounds¹⁵⁻¹⁸ and seems to have been generated by the hydrothermal activity. These compounds are not found in the unaltered sediments (for example, Fig. 3*b*)^{6,9} where the biogenic markers with the $17\beta(H)$, $21\beta(H)$ stereochemistry and various triterpenes predominate. Minor amounts of $17\beta(H)$, $21\alpha(H)$ -moretanes (C_{29} , C_{30} and C_{31}), $17\beta(H)$, $21\beta(H)$ -hopanes (C_{27} and C_{30}), *iso*-hop-13(18)-ene and $17\beta(H)$ -moret-22(29)-ene are also present in sample 7D-2B. These are precursor relics and intermediates from the thermal conversion process.

The steroidal markers consist of primarily $5\alpha(H)$, $14\alpha(H)$, $17\alpha(H)$ -steranes (20 R), with lesser amounts of $5\beta(H)$, $14\alpha(H)$, $17\alpha(H)$ -steranes (20 R) and diasteranes [mainly the $13\beta(H)$, $17\alpha(H)$ -20 R or S series]^{17,19-21}. The steranes ranged from C_{26} to C_{30} , with cholestane as the major homologue (Fig. 3*c*). The distribution pattern indicates a marine autochthonous origin and fits best with similar data for DSDP Site 477 (ref. 9). The large $5\alpha(H)$ -sterane concentration is a result of the elevated thermal stress which probably converted other steroidal compounds to these hydrocarbons⁹.

The extended diterpenoidal, triterpenoidal and steroidal indicator compounds are not detectable by GC-MS in the hydrocarbon fraction of sample 7D-4A, B. This fraction is composed of more volatile, lower molecular weight compounds.

The GC traces of the aromatic/naphthenic fractions (F2) of both samples are given in Fig. 4. Again, the hump of unresolved material is skewed to the lower molecular weight region in the case of sample 7D-4A, B. The GC-MS data indicate that the major resolved peaks are polynuclear aromatic hydrocarbons

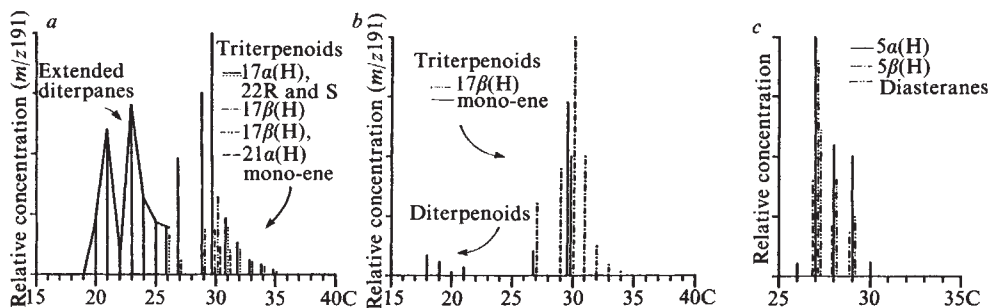


Fig. 3 Relative distribution diagrams for triterpenoids in: *a*, sample 7D-2B; and *b*, an unaltered sample, station 30G, 3.5 m subbottom⁶, and steranes in *c*, sample 7D-2B. Relative concentrations are based upon GC responses and m/z 191 and 217 mass chromatograms.

Table 3 Polynuclear aromatic hydrocarbons in dredge samples from Guaymas Basin

Compound	No.*	Formula	MW	7D-2B (ng per g bitumen)	7D-4A, B (ng per g bitumen)
Indane		C ₉ H ₁₀	118	0.5	0.4
Tetramethylbenzene	1	C ₁₀ H ₁₄	134	0.6	4.5
Naphthalene		C ₁₀ H ₈	128	4	12
2-Methylnaphthalene		C ₁₁ H ₁₀	142	8	6
1-Methylnaphthalene		C ₁₁ H ₁₀	142	6	4
Biphenyl		C ₁₂ H ₁₀	154	3	1.2
Dimethylnaphthalene		C ₁₂ H ₁₂	156	50	8
Acenaphthene		C ₁₂ H ₁₀	154	0.8	2.8
Trimethylnaphthalene		C ₁₃ H ₁₄	170	16	40
Fluorene		C ₁₃ H ₁₀	166	5	0.8
Dibenzothiophene		C ₁₂ H ₈ S	184	12	8
Phenanthrene	2	C ₁₄ H ₁₀	178	27	8
Anthracene		C ₁₄ H ₁₀	178	5	10
Fluoranthene		C ₁₆ H ₁₀	202	40	34
Pyrene	3	C ₁₆ H ₁₀	202	200	178
2,3-Dibenzofluorene		C ₁₇ H ₁₂	216	40	40
Benz(a)anthracene	4	C ₁₈ H ₁₂	228	15	16
Chrysene (triphenylene)	5	C ₁₈ H ₁₂	228	42	38
Benzo(b)fluoranthene		C ₂₀ H ₁₂	252	40	28
Benzo(k)fluoranthene		C ₂₀ H ₁₂	252	10	6
Benzo(e)pyrene	7	C ₂₀ H ₁₂	252	62	45
Benzo(a)pyrene	8	C ₂₀ H ₁₂	252	25	16
Perylene	9	C ₂₀ H ₁₂	252	45	40
1,2,5,6-Dibenz-anthracene		C ₂₂ H ₁₄	278	3	2.4
Benzo(g,h,i)perylene	10	C ₂₂ H ₁₂	276	100	24
Coronene	11	C ₂₄ H ₁₂	300	64	5

* See Fig. 4.

(PAH), another group of compounds uncommon in petroleum but ubiquitous in higher temperature pyrolysis residues (for example, smoke or from *in situ* organic matter)^{12,22,23}.

The major components that were identified and resolved are listed in Table 3. The concentrations ($\mu\text{g per g}$ of total oil) of the major analogues are of the same order of magnitude for both samples. The dominant analogues for both samples are the peri-condensed aromatic series as for example pyrene, benzopyrenes, perylene, benzopyrene and coronene. These peri-condensed structures are more reactive than their angularly condensed analogues²². Thus, they must have been expelled rapidly after genesis to the seabed for their preservation. A further indication for a pyrolytic origin is the presence of five-membered alicyclic rings (such as acenaphthene, fluorene, and fluoranthene; Table 3) which are found in all pyrolysates

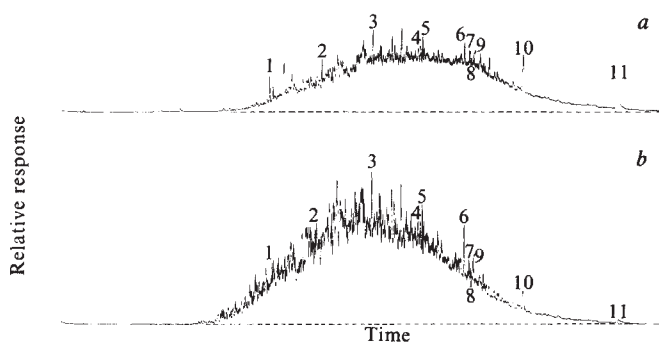


Fig. 4 Gas chromatograms of the aromatic/naphthenic hydrocarbon fractions (F2) for: a, sample 7D-2B; and b, sample 7D-4A, B. GC conditions as cited in the text. 1, tetramethylbenzene; 2, phenanthrene; 3, pyrene; 4, benz(a)anthracene; 5, chrysene; 6, benzofluoranthene; 7, benzo(e)pyrene; 8, benzo(a)pyrene; 9, perylene; 10, benzopyrene; 11, coronene.

from organic matter, because once formed they do not easily revert to peri-condensed aromatic hydrocarbons^{22,23}. Note also that this fraction contains significant amounts of toxic PAH. The benzopyrenes are major components and the highly toxic benzo(a)pyrene is present at levels of 25 and 16 ng per g of total bitumen. Perylene is present in these fractions and is also the dominant PAH in the unaltered sedimentary lipids of samples deposited in the Gulf from oxygen-minimum environments^{9,24}. Thus, the chemical composition of this fraction indicates a source from pyrolysis with rapid quenching by hydrothermal removal and subsequent condensation at the seabed.

Conclusions

The thermogenic origin of the petroleum-like organic matter in the dredge samples has been confirmed. This is based on the following chemical parameters: (1) presence of gasoline range hydrocarbons (also the odorless compounds); (2) broad distribution of hydrocarbons (hump, C₁₃–C₃₃) and presence of pristane and phytane; and (3) the relative amounts of aromatic/naphthenic (F2) and asphaltic (F3) material versus the aliphatic hydrocarbons (F1).

The indications that these oils are derived from a higher temperature pyrolysis process rather than the normal petroleum genesis window (maximum $\sim 150^\circ\text{C}$)¹² are as follows: first, the presence of peri-condensed PAH, and second, a large amount of asphaltic material and possibly the presence of various branched and in-chain olefins.

Hydrothermal migration is suggested by the following data: (1) the virtual absence of primary olefins (they were dominant components near sills at depth in for example DSDP Site 481A)⁹, which are highly reactive and are not expected to survive migration in hot fluids; and (2) the same molecular marker fingerprint as *in situ* thermogenic bitumen at DSDP Sites 477 and 477A (ref. 9). This is not a case of migration of mature petroleum from geologically old formations (>Quaternary) through the younger sequences, where it could have dissolved the indicators just discussed. Such an explanation is inconsistent with the dramatic differences in boiling range for both the aliphatic and aromatic hydrocarbon fractions (F1 and F2) of the two samples. It is more likely that a temperature gradient exists at the seabed away from the hydrothermal vent, which controls condensation and solution into ambient cold seawater of the emanating organic matter (oil). This needs further sampling in more controlled conditions.

Another indication that these oils are derived from thermal alteration of the immature organic matter (Quaternary) in the basin is found in the minor biogenic marker residues. Traces of 17 β (H), 21 β (H)-hopanes and triterpenes are present in sample 7D-2B and these compounds are not found in mature petroleum. The distribution patterns of these molecular markers are very similar to examples from samples at depth (DSDP Sites 477 and 481)⁹.

The sediments at depth (DSDP Sites 477, 478 and 481) contain large amounts of thermogenic (admixed with some original biogenic) interstitial gas^{8,10}, and also thermally derived bitumen⁹. Thus, the presence of petroliferous condensate at the seabed indicates that the plume water must contain large amounts of methane and higher molecular weight organic matter. This organic matter can represent a carbon source to microbes at the vent site, which in turn can be food for the higher fauna.

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Cloning and sequence analysis of cDNA for bovine adrenal preproenkephalin

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The nucleotide sequence of cloned cDNA for preproenkephalin from bovine adrenal medulla indicates that the precursor protein contains four copies of Met-enkephalin and one copy each of Leu-enkephalin, Met-enkephalin-Arg⁶-Phe⁷ and Met-enkephalin-Arg⁶-Gly⁷-Leu⁸, a previously undetected opioid peptide. The enkephalin and extended enkephalin sequences are each bounded by paired basic amino acid residues. Preproenkephalin may represent a multi-hormone precursor, like the corticotropin-β-lipotropin precursor.

METHIONINE-enkephalin (Met-enkephalin) and leucine-enkephalin (Leu-enkephalin) are endogenous pentapeptides with opiate-like activity which were originally discovered in the brain¹. The Met-enkephalin sequence is contained in the structure of β-lipotropin (β-LPH), which is regarded as a pituitary hormone. We have previously purified the bovine mRNA coding for the common precursor of β-LPH and corticotropin (ACTH)² and have elucidated the primary structure of this precursor protein by determining the nucleotide sequence of cloned DNA complementary to the mRNA³. Although β-endorphin, a 31-amino acid opioid peptide, which contains the Met-enkephalin sequence, has been shown to be produced from the ACTH-β-LPH precursor (alternatively designated preproopiomelanocortin) by post-translational processing, accumulating evidence indicates that Met-enkephalin itself is not derived from the same precursor (see ref. 4 for review). Furthermore, several polypeptides containing Met-enkephalin and/or Leu-enkephalin sequences, and which are not related to β-endorphin, have recently been isolated from bovine adrenal medulla^{5–11}. Thus, these polypeptides have been suggested as intermediates in the biosynthesis of the opioid pentapeptides.

In an attempt to elucidate the primary structure of the initial enkephalin precursor (hereafter referred to as preproenkephalin), we have now cloned DNA sequences complementary to the bovine mRNA coding for the precursor protein. Nucleotide sequence analysis of the cloned cDNA has revealed the whole amino acid sequence of bovine preproenkephalin.

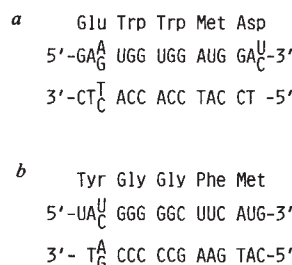
Isolation of cDNA clones and strategy of DNA sequencing

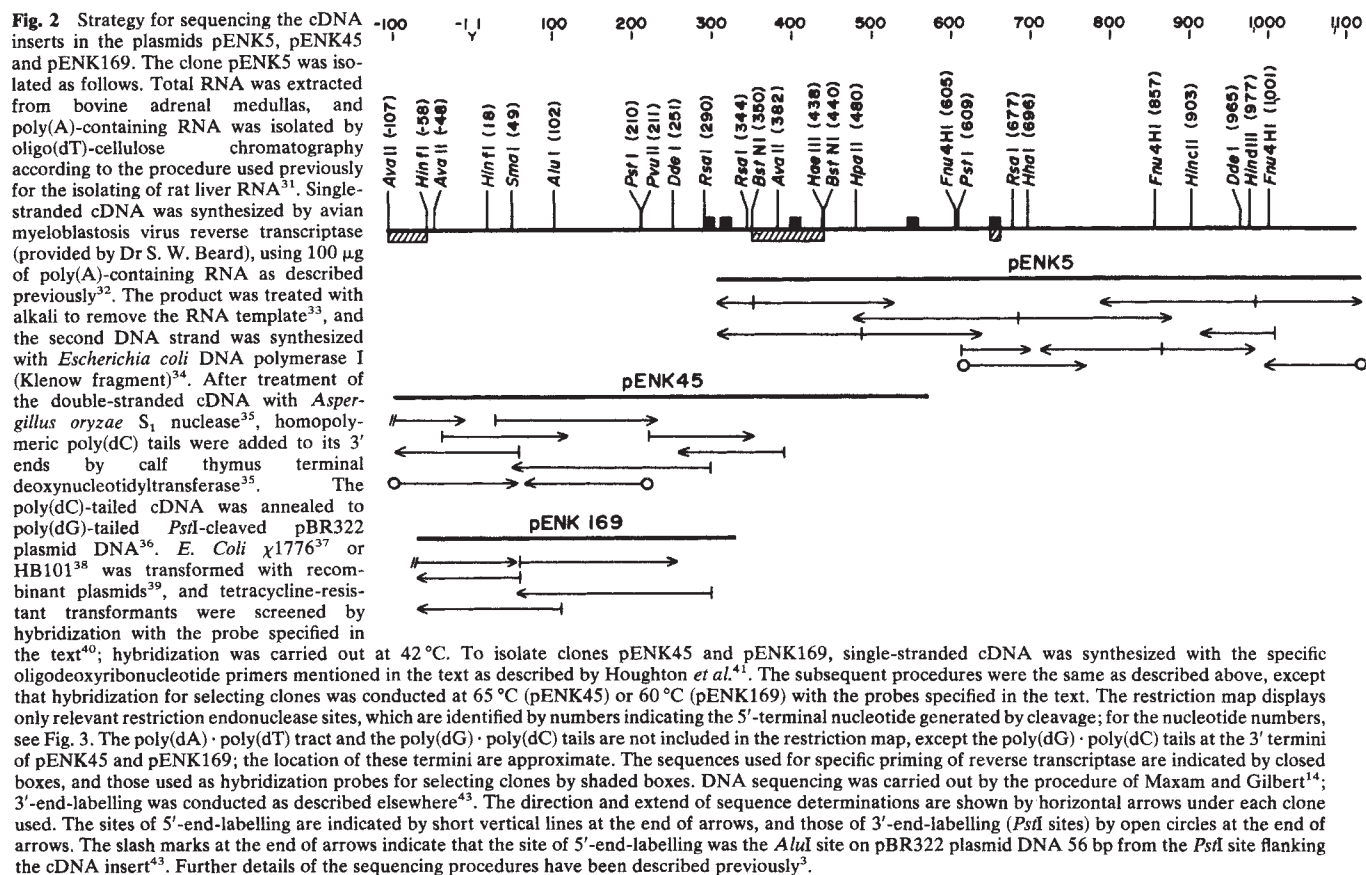
The initial approach used to clone DNA sequences complementary to preproenkephalin mRNA was to screen a library of cDNA clones by hybridization with a mixture of synthetic oligodeoxyribonucleotides, the sequences of which represented all possible sequences predicted from a known partial amino acid sequence of an enkephalin-containing polypeptide. The choice of appropriate hybridization conditions including temperature virtually eliminates the formation of mismatched duplexes without affecting the formation of perfectly matched

ones, thus ensuring the selection of desired clones. (Such a screening principle¹² would be useful for isolating cDNA clones that carry mRNA sequences present in low abundance.) After isolating cDNA clones carrying a partial mRNA sequence and determining the nucleotide sequence of the cDNA, a synthetic oligodeoxyribonucleotide primer complementary to a portion of the determined mRNA sequence was elongated by reverse transcription of preproenkephalin mRNA. The cDNA transcripts thus formed were cloned and screened by hybridization with an appropriate restriction fragment derived from initially isolated cDNA clones to yield clones carrying mRNA sequences upstream from the site corresponding to the synthetic primer used.

Poly (A)-containing RNA was isolated from bovine adrenal medullas, and double-stranded cDNA was synthesized by reverse transcription of this RNA template using oligo (dT) as primer. A cloned cDNA library was constructed by inserting the total cDNA population into the *Pst*I endonuclease cleavage site of the bacterial plasmid pBR322 with the use of poly(dG)·poly(dC) homopolymeric extensions. Two oligodeoxyribonucleotides, 5'-TCCATCCACCATTTC-3' and 5'-TCCATCCACCACTC-3' (Fig. 1a), were synthesized by the modified triester method¹³. These tetradecamers represent the only two possible cDNA sequences corresponding to the unique pentapeptide sequence Glu-Trp-Trp-Met-Asp (excluding the

Fig. 1 Synthetic oligodeoxyribonucleotides used for priming the reverse transcription of preproenkephalin mRNA and/or for probing cloned cDNA for preproenkephalin. In a, all possible coding sequences and the corresponding tetradecamers used are given for the unique pentapeptide sequence in peptide I (amino acids 217–221 of preproenkephalin). In b, the coding sequences actually found and a mixture of the corresponding tetradecamers used are shown for the four Met-enkephalin sequences composed of amino acids 97–101, 104–108, 133–137 and 182–186 of preproenkephalin. For the numbering of amino acid residues, see Fig. 3.





third nucleotide residue of the Asp codon), which is known to be a partial sequence of the enkephalin-containing polypeptides from bovine adrenal medulla, BAM-22P (ref. 8), BAM-20P (ref. 8) and peptide I (ref. 10). An equimolar mixture of the two oligodeoxyribonucleotides was labelled with ³²P at the 5' end and used as a hybridization probe to screen for recombinant plasmids carrying DNA sequences complementary to prepro-enkephalin mRNA. Nineteen hybridization-positive clones were isolated from about 190,000 transformants. On restriction endonuclease analysis, at least six of them exhibited a common restriction fragment of 68 base pairs (bp) which corresponded to the *Pst*II(609)–*Rsa*I(677) fragment containing the coding region for Glu-Trp-Trp-Met-Asp (Fig. 2), as inferred from possible coding sequences for the known amino acid sequence of peptide I¹⁰. Clone pENK5, which carried the largest cDNA insert, was subjected to nucleotide sequence analysis by the procedure of Maxam and Gilbert¹⁴ according to the strategy indicated in Fig. 2 (see Fig. 2 legend for experimental details of cloning).

Because clone pENK5 (carrying nucleotide residues 297–

1,113 given in Fig. 3) evidently did not contain the entire protein-coding sequence, a further attempt was made to isolate cDNA clones carrying the remaining mRNA sequence. For this purpose, one of the above-mentioned synthetic oligodeoxyribonucleotides, 5'-TCCATCCACCACCTC-3', the sequence of which was shown by DNA sequencing of the clone pENK5 to encode the amino acid sequence Glu-Trp-Trp-Met-Asp, was used for specific priming of the reverse transcription of prepro-enkephalin mRNA; poly(A)-containing RNA from bovine adrenal medulla served as a template. The transcripts formed by elongation of the primer were converted to double-stranded cDNA, which was cloned in plasmid pBR322 and screened by hybridization with the *Bst*NI(350)–*Bst*NI(440) fragment (Fig. 2) ³²P-labelled by nick translation¹⁵. Out of ~1,200 transformants, two hybridization-positive clones were isolated which shared an internal *Pst*I site (position 210) located upstream of the other internal *Pst*I site (position 609) present in pENK5 (Fig. 2). One of these clones, pENK45, which carried a larger cDNA insert, was subjected to DNA sequencing according to the

Table 1 Codon usage in bovine preproenkephalin mRNA

Phe	UUU	2	Ser	UCU	1	Tyr	UAU	3	Cys	UGU	1
	UUC	8		UCC	4		UAC	8		UGC	6
Leu	UUA	1		UCA	0	Ter	UAA	1	Ter	UGA	0
	UUG	0		UCG	0		UAG	0	Trp	UGG	4
Leu	CUU	4	Pro	CCU	3	His	CAU	0	Arg	CGU	1
	CUC	9		CCC	5		CAC	3		CGC	3
	CUA	4		CCA	2	Gln	CAA	0		CGA	2
	CUG	17		CCG	2		CAG	7		CGG	2
Ile	AUU	0	Thr	ACU	3	Asn	AAU	1	Ser	AGU	3
	AUC	0		ACC	6		AAC	3		AGC	7
	AUA	0		ACA	0	Lys	AAA	7	Arg	AGA	8
Met	AUG	10		ACG	1		AAG	15		AGG	2
Val	GUU	1	Ala	GCU	3	Asp	GAU	6	Gly	GGU	4
	GUC	2		GCC	5		GAC	6		GGC	12
	GUA	0		GCA	3	Glu	GAA	17 (16)		GGA	5
	GUG	3		GCG	4		GAG	15 (16)		GGG	9

Numbers next to codons indicate the numbers of amino acids using particular codons. The different numbers given for the Glu codons are due to the difference in residue 267 between the clones pENK45 and pENK169.

The translational initiation site was assigned to the methionine codon AUG at positions 1–3 because this is the first AUG triplet that appears downstream from the nonsense codon UGA (residues –96 to –94) found in frame. This assignment seems plausible because the sizes of the specific cDNA transcripts formed by reverse transcriptase-mediated elongation of

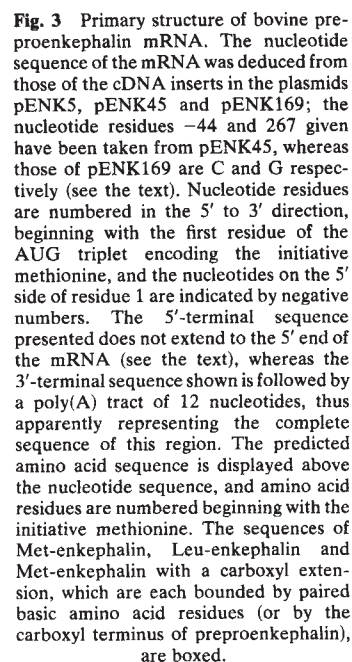
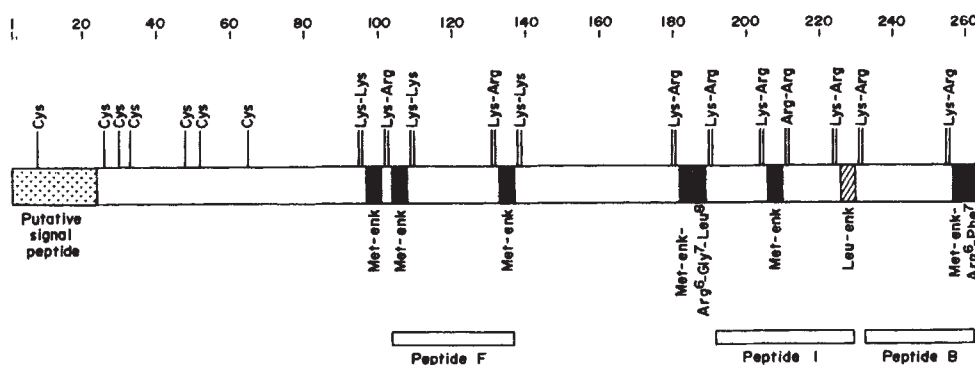


Fig. 4 Schematic representation of the structure of bovine preproenkephalin. The sequences of Met-enkephalin, Met-enkephalin-Arg⁶-Phe⁷ and Met-enkephalin-Arg⁶-Gly⁷-Leu⁸ are indicated by closed boxes, the sequence of Leu-enkephalin by a shaded box and the putative signal peptide by a stippled box. All the paired basic amino acid residues and cysteine residues are shown. Amino acid numbers (see Fig. 3) are given above. The known peptide structures, peptide F (residues 104–137), peptide I (residues 192–230) and peptide B (residues 233–263), are displayed underneath by open bars; the known peptides representing partial sequences of peptide I—peptide E (residues 206–230), BAM-22P (residues 206–227), BAM-20P (residues 206–225) and BAM-12P (residues 206–217)—are not shown.



the *Ava*II(–107)–*Hinf*I(–58) and *Ava*II(–48)–*Hinf*I(18) fragments (Fig. 2) derived from the 5′-terminal region of the clone pENK45 suggest that no more than ~65 nucleotides are missing from the cloned sequence shown in Fig. 3. Furthermore, the first 24 residues starting with the putative initiative methionine (residue 1) include many hydrophobic amino acids (19 nonpolar residues including 7 leucines). Because the signal peptide characteristic of secretory proteins¹⁶ generally contains a region rich in hydrophobic amino acids with large side chains in its central portion and terminates in a residue with a small neutral side chain¹⁷ (for example, alanine, glycine or serine), a possible site for cleavage of the signal peptide of preproenkephalin seems to be after the alanine at position 24. Assuming that the cysteine residue at position 8 is eliminated with the signal peptide, the remaining prohormone would contain six cysteine residues, all of which are located in its amino-terminal region. Other peptide hormones, such as insulin and growth hormone, contain an even number of cysteine residues which form disulphide bonds¹⁸.

The structure of bovine preproenkephalin is schematically illustrated in Fig. 4. The precursor protein is composed of 263 amino acid residues, having a calculated molecular weight of 29,786. It contains four copies of Met-enkephalin and one copy each of Leu-enkephalin, Met-enkephalin-Arg⁶-Phe⁷ and Met-enkephalin-Arg⁶-Gly⁷-Leu⁸. The amino-terminal region of the prohormone contains cysteine residues and is preceded by a putative signal peptide. There is no structure corresponding to α -neo-endorphin¹⁹ or dynorphin²⁰, both of which contain a Leu-enkephalin sequence. The sequences of Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg⁶-Phe⁷ and Met-enkephalin-Arg⁶-Gly⁷-Leu⁸ are each bounded by paired basic residues, Lys-Arg, Lys-Lys or Arg-Arg (except at the carboxyl terminus of preproenkephalin), implying that these enkephalins with and without a carboxyl extension can be formed by proteolytic processing of the precursor molecule¹⁷. It is also possible that larger enkephalin-containing peptides do not merely represent intermediates in the formation of the opioid peptides of 5–8 amino acids, but possess specific physiological functions^{7,8,10,11}. A survey of various prohormones suggests that the Lys-Arg pair may be a primary site of proteolytic cleavage^{3,17,21–23}. Assuming that this is valid for proenkephalin and that the signal peptide is cleaved after the alanine residue at position 24, the primary products formed by proteolytic processing of proenkephalin would be peptides composed of residues 25–101, 104–130, 133–179, 182–189, 192–203, 206–223, 226–230, 233–254 and 257–263; it is possible that the glycine residue at position 130 is eliminated to amidate the preceding leucine residue^{3,24}. These products include Leu-enkephalin, Met-enkephalin-Arg⁶-Phe⁷ and Met-enkephalin-Arg⁶-Gly⁷-Leu⁸. All the remaining Met-enkephalin-containing polypeptides, except the amino-terminal peptide, would have an enkephalin sequence at their amino-terminal end, as is the case for β -endorphin, α -neo-endorphin and dynorphin. This processing pattern seems to be consistent with the known opioid

activities of the larger enkephalin-containing peptides^{7,8,11}. Those with an enkephalin sequence at their amino-terminal end, such as peptide E¹¹, BAM-22P⁸ and BAM-20P⁸, but not peptide F⁹, exhibit potent opioid activity. It is conceivable that the peptide sequence next to the enkephalin portion of a large opioid peptide may modify the latter's physiological activity by conferring additional specificity for recognition of cell surface receptors, by increasing the binding affinity or by affecting its stability *in vivo*. Furthermore, the activity of a large opioid peptide may be modified by glycosylation; the sequence Asn-Ser-Ser at positions 149–151 is a possible glycosylation site²⁵.

Note that the structural organization of preproenkephalin resembles that of the ACTH- β -LPH precursor³. Both precursor proteins are composed of multiple repetitive units connected with a cysteine-containing amino-terminal sequence preceded by a signal peptide. We have previously proposed that the different component peptides of the ACTH- β -LPH precursor, acting coordinately in the central nervous system as well as in peripheral tissues, may be involved in the defence mechanism of the living organism and that the biological significance of a multi-hormone precursor may be to produce multiple peptides with coordinate functions^{3,26}. Assuming that the enkephalin-containing component peptides of preproenkephalin perform related but different physiological functions, their coexistence with catecholamines in the chromaffin granules of adrenal medullary cells^{5,27} seems to support the view that they are involved in conditions of acute stress. Thus, preproenkephalin may represent another multi-hormone precursor from which different peptides are elaborated by proteolytic processing to function coordinately in the central nervous system and in peripheral tissues.

Characteristic features of preproenkephalin mRNA

Computer analysis of the nucleotide sequence of preproenkephalin mRNA reveals that there are, in addition to the coding regions for the enkephalin sequences, many segments that seem to represent direct duplications (for example, residues 29–43 and 50–64; 121–136 and 807–822; 195–207, 462–474 and 597–609; 237–253 and 800–816; 352–367 and 421–436). This suggests that the structural gene for preproenkephalin has evolved by a series of direct duplications of certain common ancestral DNA segments followed by substitution, addition or deletion of some regions. A large number of direct duplications was also observed for mRNA coding for the ACTH- β -LPH precursor³.

Codon utilization for preproenkephalin mRNA, like other eukaryotic mRNAs²⁸, occurs nonrandomly (Table 1). Specific codon choices are preferred for some amino acids, generally reflecting a preference for G or C in the third position of the codons.

The translational termination codon UAA is followed by eight in-frame nonsense codons (residues 814–816, 898–900, 907–909, 919–921, 1,066–1,068, 1,084–1,086, 1,090–1,092

and 1,093–1,095). Two copies of the sequence AAUAAA (residues 1,064–1,069 and 1,091–1,096) are present in the 3'-untranslated region, the latter being located about 20 nucleotides upstream from the poly(A) tract. This sequence, which commonly occurs at the equivalent position of eukaryotic mRNAs²⁹, is probably involved in poly(A) addition after transcription.

After completion of this work, Gubler *et al.*³⁰ published the sequence of 45 nucleotides of bovine adrenal preproenkephalin mRNA (residues 613–657) encoding a partial sequence of peptide I. Their data, which were obtained by sequencing a

cDNA transcript formed by reverse transcriptase-mediated elongation of a specific oligonucleotide primer corresponding to the sequence Trp-Trp-Met-Asp-Tyr-Gln, differ from ours in two residues (G for residue 648 and T for residue 627).

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Molecular cloning establishes proenkephalin as precursor of enkephalin-containing peptides

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Molecular cloning and DNA sequencing have yielded considerable structural information about proenkephalin. All previously characterized intermediate peptides of the enkephalin pathway in bovine adrenal medulla have now been aligned into an unambiguous primary structure. Two basic amino acid residues serve as processing signals for release of each of the different components.

ADRENAL medulla, brain and intestinal mucosa from a variety of animal species possess enkephalin-containing polypeptides (ECPs) in addition to free enkephalin^{1–3}. Ten to twelve ECPs, ranging in molecular weight (M_r) from ~500 (0.5K) to 12K, have been purified to homogeneity from beef adrenal medulla; many of these have been completely sequenced, others only partially^{3–8}. It has been suggested that these peptides are intermediates in a biosynthetic pathway starting with 'proenkephalin'⁹. This protein has not yet been purified to homogeneity, but is known to have a M_r of 40–50K and to contain one Leu-

and six or seven Met-enkephalin sequences⁹. Such a protein precursor is a likely candidate for the primary translation product in the enkephalin biosynthetic pathway. To avoid the problem of purification and sequencing of such a large and scarce protein, we cloned and sequenced proenkephalin mRNA and deduced the primary structure of the protein from its DNA sequence. A previous report¹⁰ of the partial characterization of proenkephalin mRNA included a limited nucleotide sequence analysis which clearly established the presence of mRNA sequences encoding a previously described Met-enkephalin-

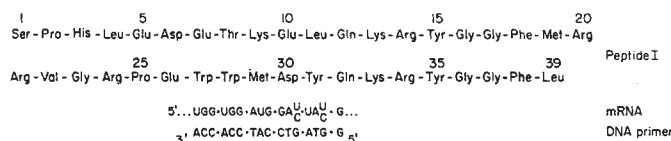


Fig. 1 The amino acid sequence and corresponding mRNA nucleotide sequence of one of the bovine adrenal ECPs (peptide I) that was used to derive the synthetic decahexamer described in ref. 10. Note that DNA sequencing has allowed us to correct two errors in the published⁴ amino acid sequence of peptide I: residue 3 has been changed to a histidine; residue 8 to a threonine. The top line shows the structure of peptide I and the middle line shows the derived mRNA sequence for the region of interest. Note the extremely low degeneracy of the codons. The synthetic decahexamer is shown at the bottom.

containing polypeptide. The mRNA was estimated to be 1,400–1,500 nucleotides long (consistent with a protein of 40–50K) and to represent 0.1% of the total poly(A⁺)RNA in beef adrenal medulla.

We now report the primary structure of most of the proenkephalin molecule as deduced from nucleotide sequences of cDNA clones. These data show unequivocally that a proenkephalin containing at least six Met-enkephalins and one Leu-enkephalin is indeed the initial product of translation in the enkephalin biosynthetic pathway.

Cloning and sequencing strategy

Total poly(A⁺)RNA from bovine adrenal medulla was transcribed into double-stranded cDNA and molecules >8S were inserted into the *Sph*I restriction site of the plasmid pBR322 by the GC-tailing technique¹¹. Recombinant plasmids containing ~300 ng of cDNA were then introduced into *Escherichia coli* RR1 by transformation, resulting in ~70,000 clones¹¹. Clones were screened by the Hanahan–Melson colony hybridization procedure¹². A synthetic decahexanucleotide was used as the radioactive probe. The sequence of this probe was predicted from the available amino acid sequence of one of the characterized ECPs (Fig. 1). This probe has also been used in the partial characterization of proenkephalin mRNA as described elsewhere¹⁰. The conditions for hybridizing the decahexamer directly to colony-bearing filters were identical to those used for probing RNA blots¹⁰. Twelve positive clones were obtained after initial screening of ~15,000 recombinants. The plasmid DNA of each of these recombinants was analysed and the largest cDNA insert (1 kilobase (kb)) was sequenced using the technique developed by Messing *et al.*¹³. This cDNA insert proved to be a copy of proenkephalin mRNA incomplete with respect to the 3' and 5' ends. Knowledge of its sequence, however, allowed us to select another of the recombinants that contained the complete 3' end and its sequence was established as above. The cloning and sequencing of the rest of the mRNA is in progress and will be reported in detail elsewhere.

Structure of proenkephalin deduced from DNA sequencing

We used recombinant DNA technology in conjunction with microprotein chemistry to establish the structure of the major part of proenkephalin. Several of the ECPs comprising proenkephalin have been isolated and their amino acid sequences determined^{3–8}. The nucleotide sequence of the partial cDNA clones has now allowed the alignment of these ECPs into an unambiguous primary structure and elucidated the amino acid sequences that link them (Fig. 2). All the ECPs previously reported from the adrenal gland are represented in the cloned

DNA sequences, which clearly establishes that proenkephalin is their common precursor (Fig. 3). The largest ECP found (12.6K; Fig. 3) actually extends beyond the 5' terminus of the largest cDNA analysed. The total size of the proenkephalin segment characterized in these combined studies is 27.3K; the initial translation product is certainly larger. Earlier experiments¹⁰ estimated proenkephalin mRNA to be 1,400–1,500 nucleotides long and we have accounted for ~1,000 of these in the sequence described here. Therefore, we can estimate proenkephalin to be no larger than ~40K. Cloning and sequencing of the 5'-terminal region of the mRNA should resolve this issue and also determine whether proenkephalin contains additional enkephalin sequences.

The sequence of the proenkephalin cDNA indicates that each isolated ECP is flanked by two basic amino acid residues (Lys-Lys, Lys-Arg, Arg-Arg), demonstrating that the ECPs purified from the bovine adrenal medulla are immediate products of proenkephalin processing rather than artefacts of isolation. The arrangement of two basic amino acid residues is known to be a typical prohormone processing signal¹⁴. Moreover, each of the seven enkephalins in the precursor is preceded immediately by these processing signals, thus the N-terminal tyrosine of each of

Glu Cys

Ser Gln Asp Cys Ala Thr Cys Ser Tyr Arg Leu Ala Arg Pro Thr Asp Leu
Asp Pro Leu Ala Cys Thr Leu Glu Cys Glu Gly Lys Leu Pro Ser Leu Lys
—|— GCT TGC ACT CTG GAA TGT GAG GGG AAA CTA CCT TCT CTC AAG
Thr Trp Glu Thr Cys Lys Glu Leu Leu Gln Leu Thr Lys Leu Glu Leu Pro
ACC TGG GAA ACC TGC AAG GAG CTT CTG CAG CTG ACC AAA CTA GAA CTT CCT
Pro Asp Ala Thr Ser Ala Leu Ser Lys Gln Glu Glu Ser His Leu Leu Ala
CCA GAT GCC ACC AGT GCC CTC AGC AAA CAG GAG GAG AGC CAC CTG CTT GCT
Lys Lys Tyr Gly Gly Phe Met Lys Arg Tyr Gly Gly Phe Met Lys Lys Met
AAG AAG TAC GGG GGC TTC ATG AAG CGG TAT GGG GGC TTC ATG AAG AAA ATG
Asp Glu Leu Tyr Pro Leu Glu Val Glu Glu Glu Ala Asn Gly Gly Glu Val
GAT GAG CTG TAC CCC CTG GAA GTG GAA GAA GAG GCA AAT GGA GGT GAG GTC
Leu Gly Lys Arg Tyr Gly Gly Phe Met Lys Lys Asp Ala Glu Glu Asp Asp
CTT GGC AAG AGA TAT GGG GGC TTC ATG AAG AAG GAT GCA GAG GAA GAT GAC
Gly Leu Gly Asn Ser Ser Asn Leu Leu Lys Glu Leu Leu Gly Ala Gly Asp
GGC CTG GGC AAC TCC TCC AAC CTG CTC AAG GAG CTG CTG GGA GCC GGG GAC
Gln Arg Glu Gly Ser Leu His Gln Glu Gly Ser Asp Ala Glu Asp Val Ser
CAG CGA GAG GGG AGC CTC CAC CAG GAG GGC AGT GAT GCT GAA GAC GTG AGC
Lys Arg Tyr Gly Gly Phe Met Arg Gly Leu Lys Arg Ser Pro His Leu Glu
AAG AGA TAC GGG GGC TTC ATG AGA GGC TTA AAG AGA AGC CCC CAC CTA GAA
Asp Glu Thr Lys Glu Leu Gln Lys Arg Tyr Gly Gly Phe Met Arg Arg Val
GAT GAA ACC AAA GAG CTG CAG AAG CGA TAC GGG GGT TTC ATG AGA AGA GTG
Gly Arg Pro Glu Trp Trp Met Asp Tyr Gln Lys Arg Tyr Gly Gly Phe Leu
GGT CGT CCA GAG TGG TGG ATG GAC TAC CAG AAA AGG TAC GGT GGC TTC CTC
Lys Arg Phe Ala Glu Pro Leu Pro Ser Glu Glu Glu Gly Glu Ser Tyr Ser
AAG CGC TTC GCC GAG CCC CTA CCC TCC GAG GAA GAA GGC GAA AGT TAC TCC
Lys Glu Val Pro Glu Met Glu Lys Arg Tyr Gly Gly Phe Met Arg Phe
AAG GAA GTT CCT GAA ATG GAG AAA AGA TAT GGA GGA TTT ATG AGA TTTAAT
CCCCCTTCCCATCAGTGAACCTGAAGCCCGACGAAGCCTCTCTGCCCGAGTGAAGACTGCTGCG
CTGGTGTGTTGATTGTCCCGAGTCGCTTGCAATTATAGTTGACTTGAGAGTCCAGATAATTAAC
ATACAACCTGAAAGCTGTGATCCAGGTTCTGTGTTCTGAGAATCTTTAAGCTTTAAATATTGGTC
TGTTGCAGCTGTCTGTTTCCATGCTCAGTTTTTTGTTATCACTTTGCTCTTATTTTTCACATAAT
GCCAATAAATGCCTACTGTGTGTAGATATACTAAAA-3'

Fig. 2 Complete nucleotide sequence and derived amino acid sequence for the 27.3K ECP. The enkephalins are underlined, with the flanking basic amino acids boxed. * Octapeptide, Met-enkephalin-Arg⁶-Gly⁷-Leu⁸; ** heptapeptide, Met-enkephalin-Arg⁶-Phe⁷ (see text). Note that the DNA sequence obtained from the clone does not cover the entire amino acid sequence of the largest ECP. The stop codon is underlined, as is the sequence AATAAA in the 3'-untranslated region of the mRNA.

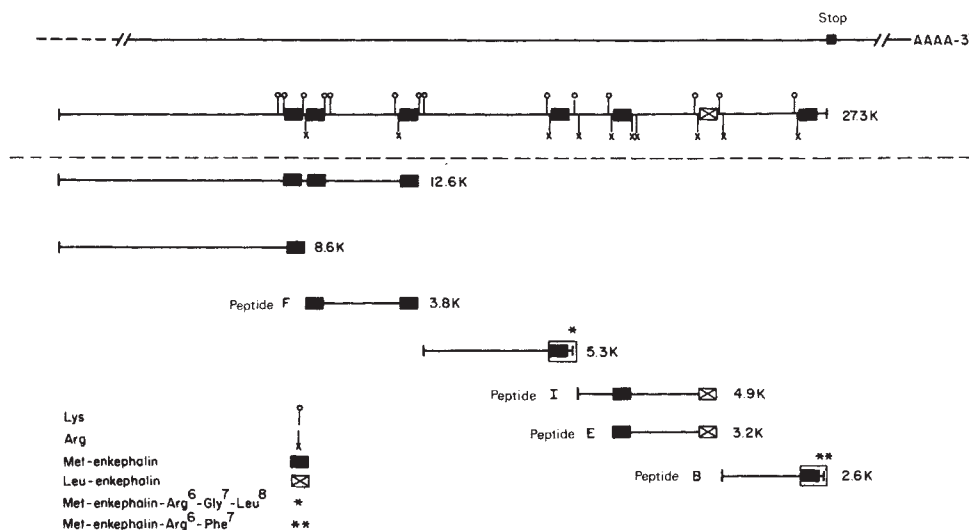


Fig. 3 Partial structure of proenkephalin and its processing. At the top is shown schematically the cDNA clone; the next line represents the deduced primary structure of the 27.3K length of proenkephalin. Below the broken line are shown the various ECPs whose structures are known. K represents M_r of 1,000.

the enkephalins, which is required for opioid activity¹⁵, is exposed in these partially processed ECPs.

Five of the enkephalins (four Met- and one Leu-enkephalin) are also immediately bounded on the C-terminal side by two basic amino acid residues, indicating that they are ultimately destined to be released as pentapeptides. As mentioned above, however, they have also been isolated as parts of large ECPs from adrenal medulla (Fig. 3). This observation indicates that differential processing of proenkephalin and ECPs may occur in the tissue.

Two of the Met-enkephalin peptides in proenkephalin are probably not processed to pentapeptides. This is indicated by the presence of additional amino acid residues between the C-terminus of the enkephalin and the dibasic amino acid processing sites, for example, the heptapeptide Met-enkephalin-Arg⁶-Phe⁷, which occurs at the carboxy-terminus of the precursor. This heptapeptide is found in the adrenal medulla evidently as a result of processing from proenkephalin at the Lys-Arg signal preceding the peptide. It had previously been placed at the carboxy-terminus of proenkephalin because a chymotrypsinlike cleavage at the phenylalanine seemed an unlikely processing event⁴. In certain assays, Met-enkephalin-Arg⁶-Phe⁷ has higher biological activity than free enkephalin¹⁶, as does peptide E⁷. Processing of proenkephalin also releases a Met-enkephalin Arg⁶-Gly⁷-Leu⁸ peptide. The presence of this octapeptide in adrenal medulla⁸ reinforces the validity of this processing mechanism. These data suggest that intermediates in the enkephalin pathway may be physiologically important and that proenkephalin yields more than free enkephalins alone.

Two technical points need further comment. We have not been able to substantiate the previous findings of a compound Leu-enkephalin-Arg⁶ (ref. 2). Due to low yields after isolation, however, this particular hexapeptide was never purified to homogeneity². DNA sequencing now clearly establishes that the Leu-enkephalin sequence is followed by a lysine. Comparison of

the partial sequence from proenkephalin mRNA reported earlier¹⁰ with the sequence given here shows two discrepancies, but these do not alter the deduced amino acid sequence. The earlier sequencing was performed on cDNA transcripts of total adrenal mRNA from several animals; the cDNA clone used here is a copy of only a single mRNA molecule. Therefore, these discrepancies could reflect some polymorphism in the proenkephalin gene among the bovine population or the presence of multiple proenkephalin genes per genome.

Two other ECPs found in hog tissue, dynorphin¹⁷ and α -neoendorphin¹⁸, are also extensions of enkephalin at its carboxy terminus and are known to have much greater biological activity than the free enkephalins. These peptides are probably products of a different ECP processing pathway as they show no apparent homology with any region of proenkephalin analysed so far except in the enkephalin sequences which they have in common. However, the adrenal heptapeptide Met-enkephalin-Arg⁶-Phe⁷ is found in brain and intestine as well as in guinea pig adrenal gland¹⁹. The tissue distribution of proenkephalin biosynthesis has yet to be determined, as is the origin of this heptapeptide in tissues other than the adrenal gland.

β -Endorphin represents another ECP, produced in the anterior pituitary gland²⁰, that has enhanced biological activity relative to free Met-enkephalin, like peptide E and the heptapeptide from proenkephalin. Indeed, the carboxyl extensions that distinguish these molecules may serve several roles, including that of stabilizing them against degradation, or in targeting.

Proenkephalin is a unique precursor in that it contains several copies of a biologically active peptide, each in a different amino acid sequence context. Complete processing of the precursor yields several enkephalins and larger ECPs. The isolation of larger ECPs that are partial products of proenkephalin processing indicates that the full potential of a multivalent precursor of this kind to yield a diversity of ECPs is probably realized *in vivo*.

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Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas

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Provirus of avian leukosis virus (ALV) are located in the vicinity of a putative cellular oncogene (c-myc) in ALV-induced bursal lymphomas. Enhanced expression of c-myc occurs in association with proviruses found in any of three configurations: (I) on the 5' side ('upstream') of c-myc in the same transcriptional orientation; (II) on the 3' side ('downstream') of c-myc in the same orientation; (III) upstream, in the transcriptional orientation opposite to that of c-myc. Thus, activation of adjacent cellular genes by retroviral DNA can involve mechanisms other than provision of a transcriptional promoter.

AVIAN leukosis viruses (ALVs) are replication-competent retroviruses which lack transforming genes but cause tumours, most commonly bursal (B lymphocyte) lymphomas, after a lengthy latent period^{1,2}. Three recent observations indicate that tumour induction by ALVs may depend on activation of cellular genes by proviral DNA, rather than on expression of viral genes. First, all bursal lymphomas are clonal populations of tumour cells containing at least one ALV provirus³⁻⁶, but solitary proviruses are often defective and many tumours are devoid of normal virus-specific mRNAs^{4,5}. Second, in most ALV-induced bursal lymphomas, proviral DNA is found in the same region of the host genome^{4,5}; Hayward *et al.* have identified this locus as *c-myc*⁷, the cellular homologue of the putative transforming gene (*v-myc*) of myelocytomatosis virus-29 (MC-29)^{8,9}. Third, many tumours contain unusual species of polyadenylated RNA which anneal with cDNA specific for the U5 domain of the long terminal repeat (LTR) in proviral DNA (cDNA_s, see Fig. 1c), but not with other viral probes^{4,5}. These novel RNAs, presumed to be initiated in proviral LTRs and extended into flanking cellular DNA, appear to contain *c-myc* sequences and are many times more abundant than the usual transcriptional product of the *c-myc* locus⁷.

Hayward *et al.*⁷ reported that 31 of 37 lymphomas contained proviral DNA linked to *c-myc*, and the structures of proviral DNA and RNA in many of these tumours were consistent with a model in which the abundant *c-myc* transcripts were initiated within a proviral LTR positioned upstream from *c-myc* in the same transcriptional orientation. Six tumours did not show enhanced expression of *c-myc*. On the basis of these findings, it was proposed that a 'promoter insertion' mechanism operates to enhance expression of cellular oncogenes such as *c-myc* during ALV tumorigenesis.

In our previous study of four defective ALV proviruses in bursal lymphomas⁴, all seemed to be located in the same region of the host genome, but two (from tumours LL1 and LL4) were in one orientation with respect to flanking DNA and two (from tumours LL2 and LL3) were in the opposite orientation. Further analysis of the tumour DNAs with probes for *c-myc* has shown that all four proviruses are located in or near the *c-myc* locus, upstream from sequences homologous to *v-myc*. The two proviruses (in LL1 and LL4) which conform to the promoter insertion model were associated, as predicted, with RNA species detectable with cDNA_s, but similar species were not observed in LL2 or LL3 in which the proviruses and *c-myc* had opposite transcriptional orientations.

The findings with LL2 and LL3 were thus inconsistent with a promoter insertion mechanism and suggested that other kinds of regulatory events might occur in the apparent oncogenic collaboration between proviral DNA and *c-myc*. We have therefore surveyed additional ALV-induced lymphomas for the

disposition of proviral DNA with respect to *c-myc* and for the composition of ALV and *c-myc*-related RNA. Our results demonstrate that at least three arrangements of proviral DNA in the *c-myc* locus are associated with enhanced levels of *c-myc* RNA: one of these arrangements is consonant with a promoter insertion mechanism but the other two—viral DNA downstream from *c-myc* in the same orientation and viral DNA upstream from *c-myc* in the opposite orientation—imply that hypotheses other than promoter insertion are required to account for the enhancement phenomena.

Strategies for studying *c-myc* activation

We have used restriction mapping procedures described by us previously⁴ (see Fig. 1c) to characterize the structure of ALV proviruses and their integration sites in 12 chicken bursal lymphomas and 4 lymphoid cell lines. Cloned restriction fragments from the *v-myc* region of MC-29 viral DNA (see Fig. 1b) served as additional hybridization reagents for determining the location and transcriptional orientation of ALV proviruses with respect to at least part of the coding domain of *c-myc*. In each case examined, an ALV provirus was situated at the *c-myc* locus. Physical maps of the interrupted *c-myc* loci from lymphoma DNA were compared with a map of the normal *c-myc* locus generated from restriction endonuclease digests of chicken DNA and from digests of a cloned fragment of chicken DNA containing most or all of the *c-myc* locus (Fig. 1a). With these methods, we have identified seven tumours and one cell line with proviruses positioned on the 5' side of *c-myc* in the same transcriptional orientation (configuration I), one tumour with a provirus on the 3' side of *c-myc* in the same orientation (configuration II), and four tumours with proviruses on the 5' side of *c-myc* but in the opposite transcriptional orientation (configuration III). We are unsure of the proviral orientation in the remaining three cell lines, each with multiple proviruses, although there is a provirus upstream from *c-myc* in every case.

We have explored the functional consequences of these arrangements of proviral and *c-myc* DNA by identifying species of polyadenylated RNA from representative tumours with hybridization probes for *myc* and for ALV sequences (particularly U3 and U5, see Fig. 1c). In some cases we also determined whether multiple hybridization reagents were annealing with separately co-migrating RNA species or with a single species. Below we present analyses of tumour DNA and RNA from samples illustrating the three arrangements of viral and *c-myc* sequences.

Configuration I: promoter insertion

EcoRI digestion of DNA from uninfected chicken cells produces a 14-kilobase pair (kbp) fragment detected by the *PstI* fragment

of cloned MC-29 DNA which served as our *v-myc* probe (referred to hereafter as *myc* probe, see Fig. 1b and Fig. 2b, lane 2). *myc* probe anneals with two fragments from *EcoRI* digests of DNA from tumour LL4—a new fragment of 3.1 kbp and the normal 14-kbp fragment (Fig. 2b, lane 1). The 3.1-kbp fragment was apparently generated by an *EcoRI* site in an ALV LTR adjacent to *c-myc*, as coincident bands were produced by hybridization to either cDNA_{3'} or cDNA_{5'} (Fig. 2b, lanes 3, 5). (Fragments of other sizes observed after hybridization of cDNA_{5'} and cDNA_{3'} to parallel digests of control DNA (lanes

4, 6) contain sequences from ALV-related endogenous provirus^{10,11}.) The *EcoRI* site in the ALV LTR is known to be in the U3 domain^{4,12}; if the same *EcoRI* restriction fragment anneals to probes for *myc*, U3 and U5, the provirus must be oriented so that it is transcribed in the same direction as *c-myc*. We have previously shown that the single ALV provirus in LL4 is highly defective, composed mostly, if not entirely, of a single LTR; hence, only two new ALV-related *EcoRI* fragments were detected with cDNA_{3'} and only one with cDNA_{5'} (Fig. 2b, lanes 3–6). Tests with several additional endonucleases have confirmed the conclusions drawn from the *EcoRI* data and have located the integration site of the defective provirus in LL4 ~0.5 kbp to the left of a *SacI* site near the 5' end of the *c-myc* sequences detectable with our *myc* probe (data not shown). Figure 2a shows schematically the arrangement of this provirus with respect to *c-myc*.

The configuration of the ALV provirus in LL4 suggests that transcription originating in the proviral LTR could proceed into *c-myc*, in the manner proposed by the promoter insertion model^{4,5,7}. The data presented in Fig. 2c substantiate this prediction. A single poly(A)⁺ RNA species of 2.5 kb was detected with cDNA_{5'} (Fig. 2c, lane 7). No other species of RNA were observed with probes for other regions of the viral genome, including U3 (Fig. 2c, lane 8), a result compatible with the truncated structure of the LL4 provirus. The *myc* probe also anneals to a 2.5-kb species of RNA from LL4 (Fig. 2c, lane 9), suggesting that the transcripts observed with cDNA_{5'} also carry *myc* sequences. Uninfected chick embryo fibroblasts also contain 2.5-kb transcripts which anneal with *myc* probe and presumably represent the normal transcription products of *c-myc*¹³ (Fig. 2c, lane 10); *c-myc* RNA of the same size and

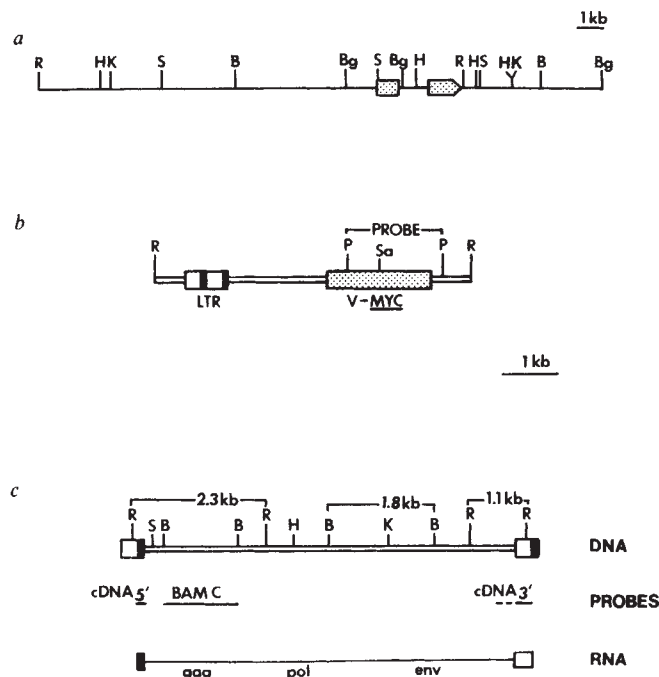


Fig. 1 a, Map of restriction endonuclease sites in the region of the chicken genome containing *c-myc*. The physical map of *c-myc* was generated using restriction endonuclease digests of chicken DNA. An identical map has been obtained from a cloned DNA fragment isolated from a recombinant phage library of chicken DNA (B. Vennstrom, manuscript in preparation). The domains homologous to *v-myc* are shown as shaded boxes and were determined using as probe a *PstI* fragment of cloned MC-29 viral DNA³⁰, which was subcloned in pBR322. This *PstI* fragment, as shown schematically in b, is 1.5 kbp long and contains, in addition to *v-myc* sequences, a short (<0.3 kbp) sequence from the *env* region of MC-29. As a result, the *v-myc* probe anneals weakly to restriction fragments of ALV-related proviral DNA containing *env* sequences, but the signal is too weak to interfere with our analyses (see Figs 2b, 3b, 4b). The *PstI* site which marks the 5' end of the *v-myc* fragment cleaves within the *v-myc* sequences (compare with b); thus, a small segment (probably <200 bp) of *v-myc*-specific nucleotides are not represented in the probe, and the shaded boxes may not represent all the *c-myc* coding sequences. The *v-myc* sequences in MC-29 are joined to *gag* sequences encoding viral structure protein³¹ generating, on translation, a fusion protein, P110^{gag-myc} (ref. 32). Thus, the structure of the *c-myc* locus is still incompletely defined, and the map does not indicate transcriptional or translational boundaries for the cellular gene. The direction of transcription of *c-myc* is indicated by the arrow-like shape of the right-hand exon and was determined by preparing restriction fragments from a *SalI* digest of the *PstI* *v-myc* fragment as probes specific for the 5' and 3' domains of *v-myc*. The length of the intervening sequence was determined by heteroduplex analysis of the cloned *c-myc* DNA fragment and MC-29 viral DNA (B. Vennstrom *et al.* in preparation). (This analysis also confirmed the transcriptional polarity of *c-myc*.) R, *EcoRI*; B, *BamHI*; S, *SacI*; K, *KpnI*; H, *HindIII*; Bg, *BglII*. b, A diagram of cloned MC-29 viral DNA. The MC-29 DNA was cloned by linearizing circular MC-29 viral DNA with *EcoRI* and inserting the DNA into λ gtWES-AB (ref. 35). The *PstI* sites (P) and *SalI* site (Sa) used to generate *v-myc*-specific probes are shown. The *v-myc* region is shown as a shaded box. c, Maps of restriction endonuclease sites in RAV-2 DNA. This map was generated as described by Payne *et al.*⁴. The LTRs, ~330 bp, are drawn as boxes at the ends of the DNA. The open box represents sequences specific to the 3' end of the viral RNA (U3) and the shaded boxes sequences specific to the 5' end of viral RNA (U5). The approximate location of viral genes are indicated on the diagram of viral RNA. 'Signature fragments', which distinguish RAV-2 DNA from proviruses endogenous to the chickens used in the present study, are marked by lines connecting the two restriction sites. Some of the probes used in our studies represent regions delineated by the labelled lines between the diagrams of viral RNA and DNA. Descriptions of each probe are included in the text and by Payne *et al.*⁴.

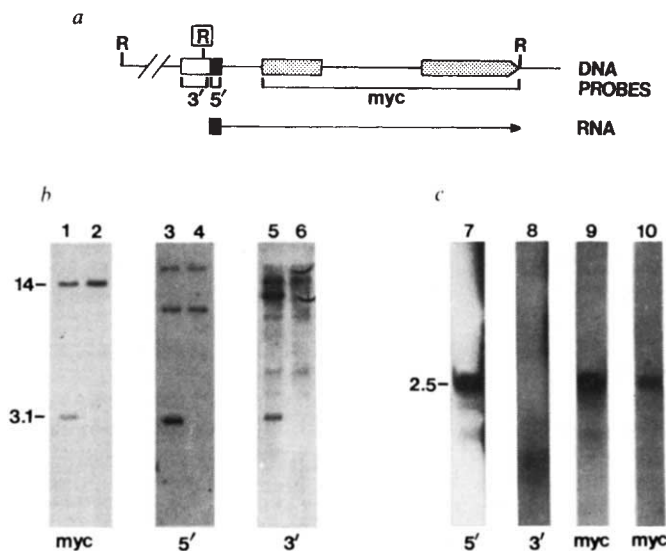


Fig. 2 Configuration I: ALV and *c-myc* DNA and RNA in tumour LL4. a, Diagram of the structure of the single ALV provirus in tumour LL4⁴ and its position and transcriptional orientation relative to *c-myc*. The symbols for the proviral LTR and *c-myc* DNA are as described in Fig. 1 legend. Cell DNA is represented as a single line. The proviral *EcoRI* site responsible for the 3.1-kbp fragment detected in b is enclosed in a box. The sequences detected by the probes used in b and c are delineated under the diagram of the DNA. The virus-specific RNA in this tumour is shown schematically below the probes. (As these transcripts are equivalent in size to normal *c-myc* transcripts, we presume the hybrid RNA is processed but we are unsure of its precise composition.) b, 5 μ g each of LL4 tumour (odd-numbered lanes) and DNA from uninfected tissue (even-numbered lanes) from the same bird were digested with *EcoRI*, electrophoresed through 0.8% agarose, transferred to nitrocellulose and annealed sequentially to the probes indicated beneath the lanes⁴. Sizes in kilobases are shown beside the lanes. c, Lanes 7–9, poly(A)⁺ RNA was prepared from the tumour as described by Varmus *et al.*¹⁷ except that frozen tissue was homogenized using a tissumizer (Tekmar) and 20- μ g samples were electrophoresed through 1.2% methyl mercury agarose, transferred to diazobenzylmethoxy(DBM)-cellulose paper³³ and annealed sequentially to the designated probes³⁴. Lane 10, 50 μ g poly(A)⁺ RNA from uninfected chick embryo fibroblasts was prepared and analysed as described above. The autoradiogram of lane 10 was exposed 8.7 times as long as the autoradiogram of lane 9.

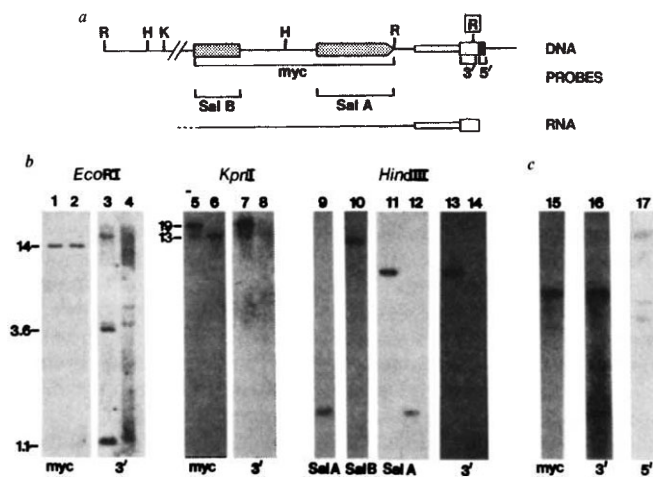


Fig. 3 Configuration II: ALV and *c-myc* DNA and RNA in tumour LL6. *a*, Diagram of the provirus and *c-myc* and the resulting transcripts in LL6 as revealed using the indicated probes in analyses shown in *b* and *c*. The symbols and the preparation of the *Sal A* and *Sal B* probes are described in Fig. 1 legend. Proviral DNA is represented as two parallel lines, cell DNA as one line. The *EcoRI* site introduced by the provirus is boxed. As explained in the text, a large deletion of cellular DNA has occurred on the 3' side of *c-myc* but the precise position and size of the deleted DNA are unknown. The *HindIII*, *KpnI* and *EcoRI* sites on the right-hand side of the provirus lie beyond the region depicted and have been omitted here. *b*, Lanes 1–8, 11–14, tumour DNA (odd-numbered lanes) and uninfected DNA (even-numbered lanes) were analysed with *EcoRI*, *KpnI* and *HindIII* using methods described in the legend to Fig. 2*b*. Lanes 9, 10, uninfected chicken embryo DNA analysed as for Fig. 2*b*. *c*, tumour RNA was analysed as for Fig. 2*c*.

abundance is present in normal bursal tissue (D. Sheiness and T. Gonda, personal communication). The concentration of *c-myc* RNA in LL4 was ~70-fold greater than in normal tissues as determined by densitometry, with adjustments for the amounts of RNA and the autoradiographic exposure times for lanes 9 and 10 (see Fig. 2 legend). Although the transcripts revealed in lanes 9 and 10 are similar in size, we do not know the precise composition of the stable species. It is likely that the transcripts in tumour LL4 have been initiated at a novel promoter provided by the ALV LTR, elongated through the *c-myc* locus and processed to remove at least some intervening sequences.

Configuration II: proviral insertion downstream from *c-myc*

EcoRI digests of DNA from tumour LL6 do not contain a novel *c-myc* fragment (Fig. 3*b*, lanes 1, 2). Hybridization with *cDNA*₃ (Fig. 3*b*, lanes 3, 4) confirmed that *c-myc* and ALV proviral sequences are not present in the same *EcoRI* restriction fragment. However, *KpnI* digestion of tumour DNA produced a new *myc*-specific fragment of ~19 kbp in addition to the normal *c-myc* 13-kbp fragment (Fig. 3*b*, lanes 5, 6). The relative intensity of the bands representing the two *KpnI* fragments implies that the uninterrupted *c-myc* allele is less abundant than the normal locus. This finding has been confirmed with other enzymes (see, for example, the analysis with *HindIII* in lane 11) and observed with several other tumours in our collection; we presume that the tumour cells in these cases are aneuploid for the chromosome bearing *c-myc*.

The annealing of *cDNA*₃ to the 19-kbp *KpnI* fragment (Fig. 3*b*, lane 7) provides evidence for an ALV provirus between the *EcoRI* site at the 3' end of *c-myc* and a *KpnI* site positioned further to the right. Confirmatory data for an insertion on the 3' side of *c-myc* were derived from *BamHI*, *BglI*, and *HindIII* digests (not shown). Further support was obtained using *SalI*–*PstI* fragments of *v-myc* as probes to distinguish between the 5' and 3' exons of *c-myc*. When uninfected chicken DNA is digested with an enzyme (for example, *HindIII*) which cleaves within the only identified *c-myc* intron (B. Vennstrom *et al.*, manuscript in preparation), the *Sal A* probe (from the 3' end of *v-myc*) detects a 1.8-kbp *HindIII* fragment in digests of normal

chicken DNA, whereas the *Sal B* probe (from the 5' end of *v-myc*) detects a 10-kbp *HindIII* fragment (Fig. 3*b*, lanes 9, 10). Using the *Sal A* probe to analyse digests of LL6 DNA, we identified a new *HindIII* fragment (6.6 kb) that also contains ALV sequences (Fig. 3*b*, lanes 11–14), confirming the position of the ALV provirus near the 3' end of *c-myc*. Further data (not presented here) show that the ALV provirus in LL6 has incurred a large deletion, leaving only the 3' LTR and ~0.7 kb of adjacent viral DNA. Furthermore, a large deletion spanning at least 15 kb of cellular DNA on the 3' side of *c-myc* has occurred in this tumour, removing the *HindIII*, *KpnI* and *SacI* sites normally proximal to the 3' exon of *c-myc* (compare with Fig. 1*a*). (This deletion explains why the apparent increments in size of *KpnI* and *HindIII* fragments in LL6 greatly exceed the size of the proviral insert.) Additional mapping results (not shown) place the defective provirus within 500 bp of the *EcoRI* site on the 3' side of the *v-myc*-related sequences in the same transcriptional orientation (Fig. 3*a*).

LL6 contains polyadenylated RNA of 3.7 kb which anneals to *myc* probe and *cDNA*₃, but not to *cDNA*₅ (Fig. 3*c*, lanes 15–17). The increase in transcript size over the size of normal *c-myc* transcripts may be partly due to ALV sequences; a probe homologous to the 3' end of *env* also hybridizes to the 3.7-kb transcripts (not shown). The concentration of this RNA was estimated to be 20 times greater than that of *c-myc* RNA in normal bursa or fibroblasts. The arrangement of the template and the size and content of the RNA suggest that transcription of *c-myc* in LL6 originates upstream from *c-myc*, perhaps at the normal initiation site, and proceeds into the ALV provirus, with termination or polyadenylation occurring in or near the end of U3 (see Fig. 3*a*). The ALV provirus has therefore altered the normal biogenesis of *c-myc* RNA, probably by replacing the *c-myc* sequences normally recognized as signals for transcript termination or polyadenylation.

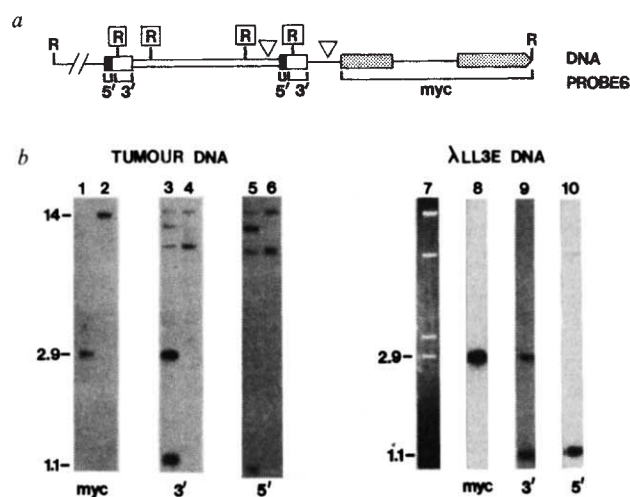


Fig. 4 Configuration III: ALV and *c-myc* DNA in tumour LL3. *a*, Diagram of the ALV provirus and *c-myc* in tumour LL3; the triangles represent deletions. The provirus in the primary tumour LL7 and its liver metastasis have a similar configuration to those in LL3 except there is no deletion in the cell DNA and the deletion within the ALV provirus is smaller. *b*, Lanes 1–6 represent analysis of *EcoRI* digests of tumour DNA (odd-numbered lanes) and uninfected tissue DNA (even-numbered lanes) using probes for *myc*, U3 and U5 as described in the legends to previous figures. Lanes 7–10 contain DNA from λ LL3E, a recombinant bacteriophage carrying *c-myc* and part of the ALV provirus from LL3 tumour DNA. Lane 7, a photograph of the ethidium bromide-stained *EcoRI* digest of λ LL3E DNA visualized by transillumination with UV light; lanes 8–10, autoradiograms of the results of sequential hybridizations with *myc* probe, *cDNA*₃ and *cDNA*₅ to cloned DNA transferred to nitrocellulose. λ LL3E and two others containing different DNA fragments from the *c-myc* region of LL3 tumour DNA were isolated from a recombinant DNA library of partial products of *Sau3A* digestion of tumour DNA cloned in the bacteriophage vector λ 1059¹⁴. Size-fractionated DNA, ~20 kb long, was selected by preparative electrophoresis of 200 μ g of digested DNA through low-melting agarose (Seaplaque)⁴. After recombinant molecules had been packaged *in vitro*³⁵ and used to infect *Escherichia coli* Q359¹⁴, recombinant phage carrying the ALV provirus were detected using *cDNA*₃ (ref. 36).

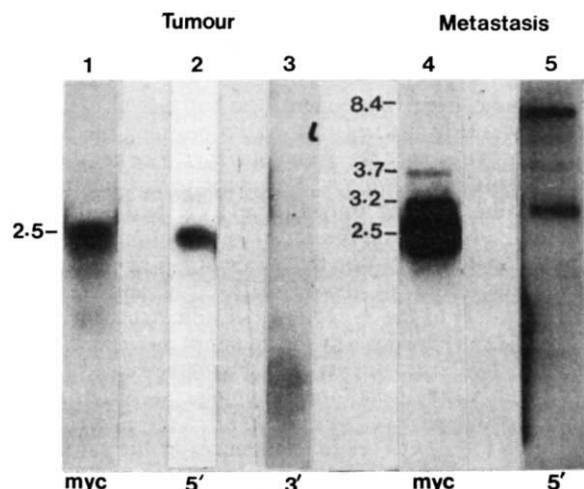


Fig. 5 Configuration III; ALV and *c-myc* RNA in tumour LL7 and its metastasis. Lanes 1–3, analysis of virus-specific RNA from bursal tumour LL7 was performed as in Fig. 2c. Lanes 4 and 5 represent an analysis of RNA from a liver metastasis from tumour LL7. 5 μ g of poly(A)⁺ RNA was electrophoresed through 1.2% formaldehyde agarose and transferred to nitrocellulose³⁷. The sequential hybridizations using the indicated probes were performed as with the DBM-filter bound RNA.

Configuration III: proviral insertion upstream from *c-myc* in the 'opposite' orientation

Hybridization of *Eco*RI-digested LL3 DNA with *myc* probe reveals a 2.9-kbp fragment in addition to the normal 14-kbp fragment (Fig. 4b, lanes 1, 2). Unlike the new *Eco*RI fragment seen in digests of LL4 DNA (see Fig. 2b), the 2.9-kbp fragment from LL3 DNA anneals with cDNA₃, but not cDNA₅ (Fig. 4b, lanes 3–6). The most likely interpretation of these data (Fig. 4a) is that the LTR adjacent to *c-myc* has assumed a transcriptional orientation opposite to that of *c-myc*. Digestion of LL3 tumour DNA with *Kpn*I and *Hind*III followed by sequential annealings with *Bam* C probe and *myc* probe confirmed the proposed orientation (data not shown). Our conclusion is further supported by previously reported evidence that the ALV proviruses in LL3 and LL4 are in opposite orientations within the same chromosomal context⁴.

We have constructed a recombinant DNA library of LL3 DNA by inserting tumour DNA partially digested with *Sau*3A into the bacteriophage vector λ 1059 (ref. 14). Three recombinant phage containing the ALV provirus and flanking cell sequences were isolated from the library. Figure 4c shows ethidium bromide-stained, *Eco*RI-digested DNA from one of our isolates (lane 7) and the results after Southern transfer and hybridization with *myc* probe, cDNA₃ and cDNA₅ (lanes 8–10). The 2.9-kbp fragment derived from the cloned DNA anneals to cDNA₃ and *myc* probe but not to cDNA₅. These data and a more detailed analysis of our three isolates (not shown) are in complete agreement with the results obtained using tumour DNA.

Three other bursal tumours (LL2 (ref. 4), LL5 and LL7) have an arrangement of ALV DNA and *c-myc* similar to that described for LL3. We previously reported that LL2 and LL3 lack polyadenylated RNA detectable with cDNA_{rep} or cDNA₅. These results, together with the conclusions from restriction mapping, imply that if the *c-myc* locus is activated by the adjacent ALV DNA in these tumours, a viral promoter is not directly responsible. To address this issue with reagents specific for *c-myc* as well as ALV sequences, we examined RNA from the primary tumour and a large hepatic metastasis of LL7. *myc* probe reveals a species of RNA in the LL7 bursal tumour migrating at 2.5 kb (Fig. 5, lane 1). Surprisingly, this RNA also seemed to react with cDNA₅ (Fig. 5, lane 2) but not with probes for other regions of the genome, including cDNA₃ (Fig. 5, lane 3). Because our cDNA₅ probe is strand specific (it only detects RNA with the same polarity as the viral RNA genome), the

orientation of the ALV provirus and *c-myc* in tumour LL7 precludes the possibility that ALV U5 sequences from this provirus are physically joined to a transcript of the *c-myc* coding strand (see Fig. 4a). This type of transcript might be produced by a subset of cells in LL7 containing a provirus upstream from *c-myc* in the same orientation. These cells could produce high levels of transcripts with U5 sequences joined to *c-myc* sequences and remain undetected in our DNA analyses if they represented <10% of the total tumour cell population.

In an attempt to isolate a homogeneous population of tumour cells, we examined a liver metastasis from the LL7 tumour. Metastases are often clonal expansions of single tumour cells^{4,15}; restriction enzyme analyses of the metastasis DNA (not shown) indicated that the arrangement of ALV and *c-myc* DNA in the LL7 metastasis was identical to that in the predominant cell population in the primary tumour. However, the major 2.5-kb species of RNA from the metastasis which annealed to the *myc* probe did not react with cDNA₅ (Fig. 5, lane 5) or anneal to probes for other regions of the ALV genome (data not shown).

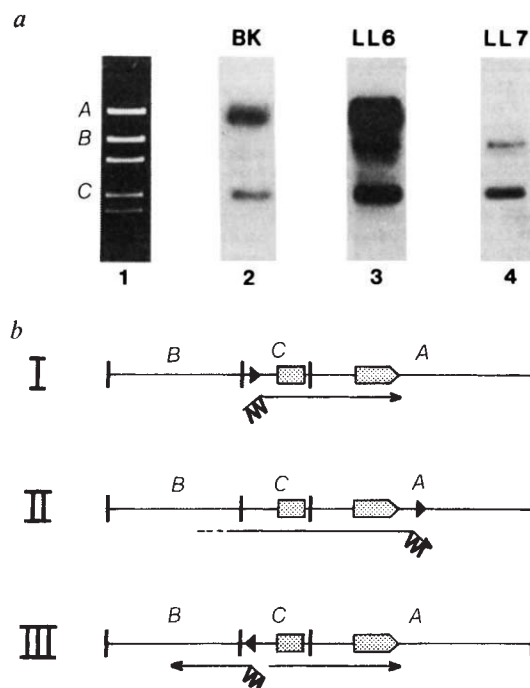


Fig. 6 Tests for linkage of ALV and *myc* sequences in tumour RNAs. The sandwich blot procedure of Dunn and Hassell¹⁶, modified as described below, was used to assess physical linkage of ALV sequences and *myc* sequences in RNA prepared from BK 4484A cell line RNA (lane 2), LL6 RNA (lane 3) and LL7 liver metastasis RNA (lane 4). The 10-kbp *Bam*HI fragment containing *c-myc* (see Fig. 1a), obtained from a digest of the recombinant DNA described in Fig. 1a legend, was subcloned into pBR322 (D. Sheiness, unpublished). 1 μ g of this plasmid, pCMC-2, was digested with *Bam*HI and *Bgl*II and electrophoresed through 1.2% agarose. The DNA was detected as described in Fig. 4 legend, then transferred to nitrocellulose. 5 μ g of poly-(A)⁺ RNA was resuspended in 200 μ l of an annealing mix consisting of 50% formamide, 0.5 M NaCl, 20 mM PIPES pH 6.8, 5 mM Na₂EDTA, 0.4% SDS, 250 μ g ml⁻¹ poly(A), 2 $\times$ Denhardt's solution³⁸, 200 μ g ml⁻¹ carrier yeast RNA. This solution was applied to the filter-bound DNA and incubated for 24 h at 41 $^{\circ}$ C. The filter was washed twice at 53 $^{\circ}$ C for 15 min, each with 0.1 $\times$ SSC, 0.1% SDS. The filter was blotted dry and annealed to cDNA₅ or cDNA₃ as described by Quintrell *et al.*³⁴. This filter was washed twice for 1 h each time at 37 $^{\circ}$ C with 0.1 $\times$ SSC, 0.1% SDS, dried and autoradiographed. Lane 1, photograph of ethidium bromide-stained *Bgl*II-*Bam*HI digest of pCMC-2 DNA. The bands labelled A, B and C result from the *c-myc* insert and are shown schematically in b. The other bands consist of pBR322 DNA sequences. Lanes 2, 4, filters annealed to cDNA₅. Lane 3, filter annealed to cDNA₃. b, Diagrams of transcriptional patterns in configurations I, II and III based on sandwich blots shown in a and analyses of tumour RNA from BK cell line (not shown), LL6 (Fig. 3c) and LL7 (Fig. 5, lanes 4, 5). The restriction sites are labelled as in Fig. 1a and the fragments designated A, B and C in a are shown. The arrowhead marks the position of the ALV provirus and indicates its transcriptional orientation. The transcriptional pattern of each configuration is shown as an arrow below the diagram. The inclined tail of an arrow marked with a serrated line indicates ALV U5 sequences detected with cDNA₅ in configurations I and III, and ALV U3 sequences detected with cDNA₃ in configuration II.

Thus, the metastatic process seemed to isolate a population of tumour cells with an ALV provirus upstream from *c-myc* in the opposite orientation and with enhanced expression of *c-myc* in the absence of a fused transcript. The analysis of metastasis RNA in Fig. 5 revealed minor transcripts (3.7 and 3.2 kb in lane 4; 8.4 and 3.2 kb in lane 5) which were apparently absent from the primary tumour. However, long autoradiographic exposures of the filters carrying primary tumour RNA revealed faint bands corresponding to these species (not shown). The 8.4- and 3.2-kb transcripts revealed by cDNA₅ represent normal ALV mRNAs and imply the presence of a few cells which harbour non-defective ALV proviruses, either superinfected metastatic lymphocytes or infected normal liver cells. The coincidence of the 3.2-kb transcript detected by the probes in Fig. 5, lanes 4 and 5, can be attributed to co-migrating transcripts (see below).

The *myc* and ALV sequences are covalently joined in RNA from tumours with configurations I and II but not III

Using the 'sandwich blot' procedure of Dunn and Hassell¹⁶ (Fig. 6), we assessed whether ALV U5 sequences were physically linked to *myc* sequences in tumour transcripts. *Bam*HI-*Bgl*II double digests of cloned chicken *c-myc* DNA were electrophoresed (Fig. 6a, lane 1) and transferred to nitrocellulose. Unlabelled tumour RNA was annealed to the filter-bound DNA, the filters were washed, and RNA containing ALV U5 or U3 sequences detected by hybridization to labelled cDNA₅ or cDNA₃. An autoradiographic signal in a position corresponding to one of the *Bam*HI-*Bgl*II restriction fragments indicates physical linkage in RNA of sequences homologous to both the restriction fragment and the ALV LTR. When we used RNA from a tumour cell line (BK 4484A) containing an ALV provirus upstream from *c-myc* in a parallel orientation (configuration I), we observed bands corresponding to fragments A and C but not B when cDNA₅ was used as a radioactive probe (Fig. 6a, lane 2). This result proves that these cells contain transcripts consisting of ALV U5 sequences physically linked to the *c-myc* sequences in fragments C and A. This conclusion is shown schematically in configuration I of Fig. 6b and conforms to the promoter insertion model.

Figure 6a, lane 3, demonstrates that ALV U3 sequences and *myc* sequences are physically linked in RNA from tumour LL6. The signal observed with cDNA₃ at the position of fragment B locates the 5' end of these transcripts at least 1 kb upstream from the *Sac*I site marking the 5' end of the *c-myc* sequences detected by our probe (see Figs 1a, 6b). If these transcripts originate at the normal *c-myc* promoter, these data place that promoter to the left of the *Bgl*II site which defines the right end of fragment B (see configuration II in Fig. 6b). Studies of three tumours (LL4, LL5 and LL7) and one cell line (BK 4484A) showed that each provirus is integrated to the right of this *Bgl*II site. Thus, proviruses integrated 5' to *c-myc* in configurations I and III (see below) would be positioned between the normal promoter and the *myc* sequences that we have detected at increased concentrations in tumour RNA; this could mean that a secondary initiation site is used for *myc* RNA in these cases. We cannot, however, exclude other possibilities; for example, the transcripts in tumour LL6 may be initiated aberrantly, thus obscuring the location of the true *c-myc* promoter, or viral sequences could be transcribed in configuration III and removed from RNA by splicing.

RNA from the LL7 metastasis contains transcripts with U5 joined to sequences in fragments C and B but not A (Fig. 6a, lane 4). Because restriction mapping data (not shown) place the ALV provirus in LL7 5' to the *c-myc* sequences detected by our probe, the sandwich blot indicates that transcription probably originates in the proviral LTR and proceeds through the region represented in fragment C and into that contained in fragment B. It also confirms that ALV U5 sequences are not joined to the identified *c-myc* coding sequences in transcripts (Fig. 5). Thus, the 3.2-kb RNA species revealed in Fig. 5, lanes 4 and 5,

probably represents co-migration of *env* mRNA and a minor *c-myc* transcript containing no ALV sequences.

The ALV provirus can act as an insertional mutagen

We have assessed the relative configurations of ALV proviruses and *c-myc* in 12 ALV-induced bursal lymphomas and 4 cell lines derived from ALV-induced tumours. Each tumour or cell line harboured an ALV provirus situated in the region of *c-myc*, and, in every case examined (five tumours and four cell lines), this insertion was associated with levels of *myc*-containing transcripts elevated 20–100-fold over levels of *c-myc* transcripts in uninfected bursa. These data further support the proposal⁷ that ALV exerts its oncogenic effects by increasing the expression of *c-myc*.

Based on the structures of *c-myc* DNA and RNA in their tumours, Hayward *et al.*⁷ have argued that the increased level of *c-myc* transcription is uniformly due to the insertion of an ALV promoter upstream from the gene, thus subverting the expression of *c-myc* by supplying a promoter more efficient than its own. In contrast, we have shown that ALV proviruses can assume three different configurations with respect to *c-myc*, and that each configuration is associated with increased transcription of *c-myc*. We have been unable to identify a significant divergence in experimental protocol that might explain the differences between our results and those of Hayward *et al.* In view of our results, we propose that the ALV provirus (specifically the LTR) can act as an insertional mutagen capable of enhancing the expression of a cellular gene, *c-myc*, by as yet unknown mechanisms, one aspect of which may be reflected in promoter insertion.

The belief that these phenomena are instrumental in lymphomagenesis is founded primarily on the extraordinary frequency with which proviral insertions in the *c-myc* locus and high levels of *c-myc* RNA are encountered in ALV-induced bursal tumours. However, the techniques used thus far have not identified the stage of tumour induction at which *c-myc* is affected, nor have they fully assessed the possibility that sequential rearrangements have occurred at the proviral integration sites within the *c-myc* locus. (In fact, deletions affecting proviral or flanking cellular sequences have been encountered at a much greater frequency in this experimental context^{4–6} than in others (see ref. 17).) In addition, transformation experiments with DNA from bursal lymphomas indicate that oncogenic loci devoid of viral or *c-myc* sequences may also be activated during the neoplastic process^{18,19}.

The transcriptional enhancement exerted by the ALV provirus during leukaemogenesis constitutes one of two possible mutagenic effects on the host genome following retroviral DNA insertion: proviruses can also inactivate host genes. The abolition of *v-src* expression due to the insertion of Moloney murine leukaemia virus (Mo-MLV) DNA into a Rous sarcoma virus provirus has recently been observed in culture¹⁷. Also, the mouse genotype *dilute* may be due to an endogenous MLV provirus inserted at a coat colour-determining locus²⁰. The mutagenic properties of retroviral proviruses, as well as structural features²¹, are shared with transposable elements found in bacteria and yeast. Although transposable elements have usually been reported to inactivate genes when they induce mutations²², this probably reflects the genetic selection used; IS2 in bacteria²³ and Ty1 in yeast^{24,25} also alter the expression of adjacent genes in a positive manner by mechanisms still incompletely defined.

The mechanism of transcriptional enhancement

The mechanism of ALV-induced enhancement of *c-myc* expression also remains obscure. However, two general, non-exclusive proposals can be considered. First, the increase of *c-myc* expression in each of the three observed configurations

may reflect properties of *c-myc*. *c-myc* could be under the control of *cis*-acting regulatory elements either 5' or 3' to the gene. Integration of ALV DNA into these sequences could then release *c-myc* from their influence. Experiments of Cooper *et al.*²⁶ suggest that *cis*-acting sequences regulate expression of endogenous chicken proviruses, and *cis*-acting regulators on the 3' side of a gene have been reported for the mating type locus in yeast²⁷ and the fetal globin genes in humans²⁸. A second, more likely proposal postulates that enhancement reflects a distinctive property of the provirus rather than a property of *c-myc*. For example, the provirus could increase the accessibility of *c-myc* to the host cell's transcriptional machinery by altering the nucleosome positions in the region of *c-myc*. This could account for increased *c-myc* expression in each configuration. Alternatively, the mechanism of enhancement could be unique for each configuration. The proviral LTR could act as a *c-myc* promoter if inserted in the correct orientation upstream from *c-myc*. If inserted in the proper orientation downstream from *c-myc*, the provirus could act to stabilize the *c-myc* transcripts. If inserted upstream from *c-myc* but in an opposite orientation, it could affect chromatin structure or bind accessory transcriptional factors in ways which might increase transcription in both directions.

The hypothesis that a retroviral LTR can enhance the expression of a linked gene may find support in experiments which introduce selectable genes into cultured cells either as a calcium phosphate precipitate or by microinjection. The Moloney murine sarcoma virus LTR markedly augments the frequency of morphological transformation of NIH-3T3 cells by a restriction fragment containing the putative transforming gene of murine sarcoma virus (*v-mos*). This augmentation occurs

when the LTR is positioned on either the 5' or 3' side of the viral oncogene²⁹. When the LTR has been positioned downstream from *v-mos* with the same transcriptional polarity, transcripts which proceed through *v-mos* and terminate in the U3 region are observed in the transformed cells (T. Wood and G. Vande Woude, personal communication). This situation is analogous to the transcriptional pattern found in tumour LL6. Placement of the Rous sarcoma virus LTR at either the 5' or 3' end of a complete herpes simplex virus thymidine kinase (HSV-*tk*) gene, including the true HSV-*tk* promoter, enhances the number of HSV-*tk*-positive colonies after microinjection into *tk*⁻ mouse L cells (P. Luciw and M. Capecchi, personal communication). It is unclear whether the effect of the retroviral LTR in these cell culture experiments is to increase transcription of the adjacent genes.

In conclusion, we have proposed that the ALV provirus contains a previously unknown ability, independent of its configuration, to affect the transcriptional activity of adjacent cellular DNA. The transcriptional enhancement provided by the ALV provirus suggests that novel mechanisms may control the expression of eukaryotic genes.

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LETTERS TO NATURE

Occurrence of the 3.3- μ m feature in galaxies

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Many emission features have been discovered, in dusty ionized regions such as planetary nebulae or H II regions, with wavelengths longer than 2 μ m (ref. 1). Some are gaseous emission lines but many that are too broad to be due to single atoms are attributed to emission from dust grains in or near the ionized gas. The feature at 3.28 μ m ('the 3.3- μ m feature') has been observed in H II regions^{2,3} in planetary nebulae⁴ and in par-

ticular, in the following extragalactic objects: 3C273 (ref. 5) (quasar), NGC4151 (ref. 6), M82 (ref. 7), NGC253 (ref. 8), NGC2903 (ref. 9), NGC5195 (ref. 9) and NGC6946 (ref. 9). We report here new measurements which contradict some of these results. In particular, we find the feature in the late-type galaxy M83 but do not observe it in either the Seyfert galaxy NGC4151 or the quasar 3C273.

The mechanism that produces the line is thought to be due either to fluorescence of the grains in a UV radiation field¹⁰ (but this requires very high efficiencies) or to the heating of very small grains to high temperatures (several hundred degrees)¹¹. The presence of the feature in extragalactic objects such as late-type galaxies is not surprising because these objects contain many H II regions. Measures of equivalent width of the feature will indicate the chemical composition, once studies of galactic objects have established the exact nature of the mechanism. In objects such as Seyfert galaxies and quasars, however, the presence or absence of the 3.3 μ m feature is important as it is one

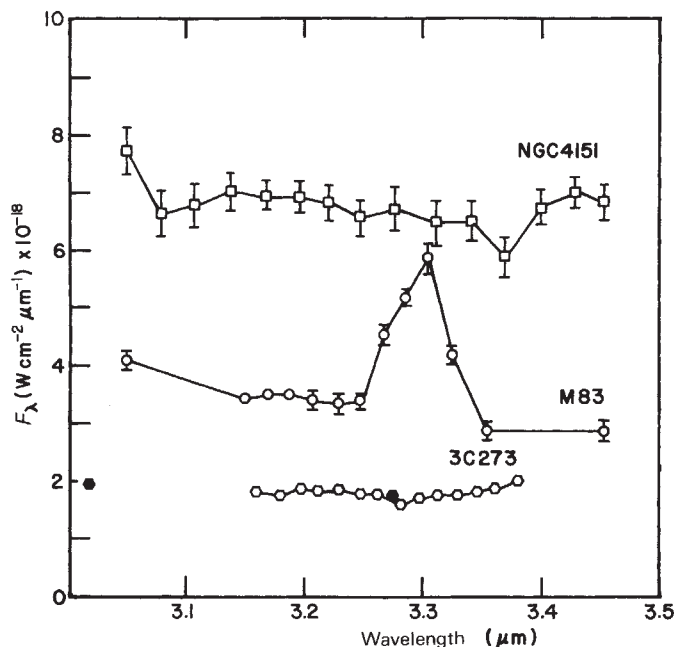


Fig. 1 The circular variable filter spectra of the three objects: NGC4151, M83 and 3C273. The wavelengths are rest wavelengths and broad-band filter measurements are also plotted for the quasar 3C273 as solid symbols. The error bars are ± 1 s.d.

of the few unambiguous dust indicators available to settle the question of the presence of dust grains in the nuclei of active galaxies.

As part of a programme to observe a larger number of objects, we have measured the spectra of 3C273, M83 and NGC4151 in the region of the local rest wavelength of the 3.3- μ m feature. This was carried out on the nights of 21, 22 and 23 May (UT) 1981, one object being measured each night. In addition, NGC4151 had been observed on 12 April 1981. During these nights the sky was clear above Mauna Kea summit and the visibility good. The telescope and instrumentation used were the UK Infrared Telescope (3.8 m) with an InSb CVF spectrophotometer (1% resolution). Apertures and chopper throws respectively were 7.3 and 13 arc s for 3C273, 10.8 and 18 arc s for M83, and 14.4 and 18 arc s for NGC4151. The wavelengths at which measurements were taken were spaced at 0.02- μ m intervals near to the position of the feature and a few more widely spaced points were also measured. In addition broad-band data were obtained. At each observed point several 10-s integrations were made and total integration times were 5,600 s for 3C273, 4,820 s for M83 and 5,640 s for NGC4151. The spectra are shown in Fig. 1, where the flux density F_λ is plotted against the rest wavelength. For both 3C273 and M83 two spectra were averaged, while for NGC4151 the April data are shown.

The April data on NGC4151 are the more extensive in wavelength and of better signal-to-noise ratio. These data set a 3σ upper limit of 8% of continuum on line height above the continuum (while the May data set a poorer upper limit of 23%). For 3C273, the corresponding limit is 9% of continuum. These limits are conservative, being based on the assumption of a flat baseline, and the spectral profile of the CVF. If the continuum outside the region of the line were to be slightly curved this would reduce the upper limits by a factor of ~ 2 . These limits should be compared with previously^{5,6} suggested values of 30% and 40% respectively. Although both 3C273 and NGC4151 are variable by a few tens of per cent, our continuum levels are sufficiently close to, and also fainter than, the previously measured continuum levels, and this cannot cause such a discrepancy.

In M83, a late-type galaxy with H II regions in its nuclear region¹², the 3.3- μ m feature is clearly seen in Fig. 1. Equally

clearly, at the levels discussed above, the feature is not present in either the Seyfert galaxy NGC4151 or the quasar 3C273. The excellent transparency in the 3- μ m region at the summit of Mauna Kea and high instrumental instantaneous signal-to-noise ratios mean that previous detection of the 3.3- μ m feature must be considered suspect. This experiment shows no evidence for dust in the Seyfert galaxy NGC4151 or the quasar 3C273, but clearly demonstrates that the dust in M83 and in H II regions in our Galaxy share similar properties.

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γ -Quanta capture by magnetic field and pair creation suppression in pulsars

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The theory¹ that electron-positron pairs in magnetospheres of pulsars are created by the interaction of γ -quanta with the magnetic field B is the basis of the modern theory of pulsars^{2,3}. When considering the process $\gamma + B \rightarrow e^+ + e^- + B$ in pulsars it is usually accepted that the γ -quanta produced by the curvature irradiation of the primary particles are emitted tangentially to the curved line of force of magnetic field and propagate directly. As they do so the pitch angle between the photon wave vector K and the magnetic field B increases until a certain value ψ_1 when the absorption of the photon through pair creation becomes possible. We emphasize here that if the resonant behaviour characteristic of the vacuum polarization in a magnetic field^{4,5} is considered this picture changes considerably. Namely, the trajectories of γ -quanta are strongly bent towards the direction of the magnetic field if the latter is sufficiently intense and at the same time the creation is suppressed. We also discuss the effect this may have on the energy yield of pulsars.

In a medium possessing a space-non-uniform refraction index, to which a strong magnetic field with curved lines of force in a vacuum is an example, a photon must propagate along a curved path. However, in this case the path would be curved negligibly as long as the traditional expression⁶ for the refraction index is used which differs from unity only by $\sim 10^{-5}$ (for the fields $B \sim 10^{12}$ G). The expressions given in ref. 6, as well as those in later publications, however, do not cover the resonant situation which occurs in the vicinity of the pair creation threshold. This resonant situation has been examined^{4,5} on the basis of the first off-mass-shell calculation of the polarization operator in an external magnetic (with electric, as well) field outlined in ref. 7 for arbitrarily strong fields. It was found that the dispersion curve deflects from its conventional shape $\omega/c = |K|$ which survives only in the region far away from the resonance (ω is the frequency). The resonant dependence of the component $K_{||}$ of the photon wave vector parallel to the

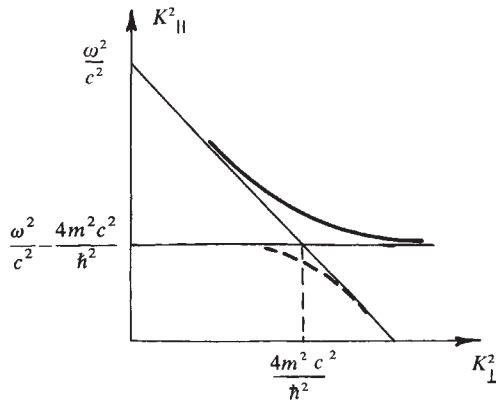


Fig. 1 Two disconnected branches of the dispersion curve of the photon in a magnetic field.

magnetic field $\mathbf{B}$ on its component $K_\perp$ across $\mathbf{B}$ with ω kept fixed is shown in Fig. 1. Part of the dispersion curve near the first threshold

$$(K_\parallel^2)_{\text{thr}} = \frac{\omega^2}{c^2} - \frac{4m^2c^2}{\hbar^2} \quad (1)$$

is shown as the solid line in Fig. 1. For $K_\parallel^2 \leq (K_\parallel^2)_{\text{thr}}$ electrons and positrons are virtually created. Other thresholds, if the value of ω is large enough, are located between the lines $K_\parallel^2 = (K_\parallel^2)_{\text{thr}}$ and $K_\parallel^2 = 0$. The solid dispersion curve near the threshold is given by the relation

$$K_\parallel^2 = \frac{\omega^2}{c^2} + f(K_\perp^2) \quad (2)$$

where

$$f(K_\perp^2) = -\frac{4m^2c^2}{\hbar^2} - \frac{2}{3} \left(K_\perp^2 - \frac{4m^2c^2}{\hbar^2} \right) + [((\phi^2 + g^3)^{1/2} + \phi)^{2/3} + ((\phi^2 + g^3)^{1/2} - \phi)^{2/3}] \frac{m^2c^2}{\hbar^2} \quad (3)$$

Here

$$g = \frac{1}{3} \left(\frac{\hbar^2 K_\perp^2}{m^2 c^2} - 4 \right), \quad \phi = \alpha \frac{B}{B_{\text{cr}}} \exp \left(-\frac{K_\perp^2 c \hbar}{2eB} \right)$$

where

$$\alpha = 1/137, \quad B_{\text{cr}} = \frac{m^2 c^3}{e \hbar} \approx 4 \times 10^{13} \text{ G}$$

Near the threshold (1) both $K_\perp^2$ and the refraction index tend to infinity. This singularity may be traced back to the fact that the spectra of electron and positron in magnetic field are partially discrete. The infinite behaviour of $K_\perp^2$ is just what we described as the resonance. Away from the threshold the dispersion curve in Fig. 1 approaches the sloping line $\omega^2/c^2 = K_\perp^2 + K_\parallel^2 = \mathbf{K}^2$. The dashed line in Fig. 1 shows the real part of the dispersion curve in the region of absorption. The frequency here has an imaginary part which is responsible for pair creation. The analytical expressions for the real and imaginary parts of the dispersion curve in this region are given elsewhere⁵. Note that above the horizontal threshold line in Fig. 1 no pair creation by the photon is possible. Figure 1 relates to the case when the magnetic field is constant in magnitude and direction. The stronger the field, the larger is the gap between the two branches of the dispersion curves in Fig. 1:

$$\Delta K_\parallel \sim \left(\frac{\alpha B}{B_{\text{cr}}} \right)^{2/3} \frac{mc}{\hbar} \exp \left(-\frac{4B_{\text{cr}}}{3B} \right)$$

The flattening of the dispersion curve (2) near the threshold causes the group velocity component across the field $v_\perp = d\omega/dK_\perp$ to tend to zero when $K_\parallel^2 \rightarrow (K_\parallel^2)_{\text{thr}}$ from inside the

region $K_\parallel^2 > (K_\parallel^2)_{\text{thr}}$, the energy of the photon thus being carried along the magnetic field. Previously a constantly-directed magnetic field was considered and this channelling effect discussed as a refraction on its edge^{4,5}. With the present knowledge of the pulsar phenomena, however, it is more reasonable to consider a constant magnetic field B , but with curved lines of force, and a photon which appears inside it and is directed under a certain initial pitch angle

$$\psi_0 = \tan^{-1} \frac{K_\perp(0)}{K_\parallel(0)} < \psi_1$$

with respect to the line of force. Assuming that the curvature radius of the line of force is much greater than the wavelength and other characteristic length parameters, we may also apply the above dispersion pattern to the present case of non-uniform magnetic field. Because the dispersive properties of the 'medium' are directionally non-uniform but constant in time, the frequency of the photon is conserved whereas its wave-vector components are not, and the photon 'travels' along its dispersion curve in Fig. 1. Classically one allows for the possibility that the photon may travel along the sloped straight line in Fig. 1, which is the empty space dispersion law. In this case the path of the photon in space is also straight. After the photon reaches the threshold line in Fig. 1 (when the pitch angle reaches the value $\tan \psi_1 = (2mc/\hbar)(\omega^2/c^2 - 4m^2c^2/\hbar^2)^{-1/2}$, and crosses it) the pair creation becomes allowed kinematically.

However, as long as the dispersion law (2) may be taken seriously (restrictions will be discussed below), the photon travels along the true dispersion curve (solid line in Fig. 1) and never crosses the threshold. Its frequency remains real and the pair cannot be created in the experiment under consideration. This does not stop a photon creating a pair if the former has been prepared below the threshold line, for example, has appeared with the pitch angle exceeding ψ_1 . The present situation is analogous to that known in the theory of polaritons⁸. Instead of creating the pair, the photon, in accordance with the above comment on the vanishing of $v_\perp$, should turn in the space along the magnetic field when approaching the threshold.

To describe the trajectory of the photon we restrict ourselves to the case of magnetic field whose strength is constant and lines of force are concentric circles. These simplifying assumptions allow us to solve the refraction problem analytically and produce a model sufficiently representative to allow the situation in the more realistic dipole magnetic field to be assessed. Within geometrical optics, the law of refraction may be derived using the fact that the photon travels in a medium whose refraction index is different in each spatial point depending on the direction of the magnetic field in it. For the special case under consideration and the photon moving away from the centre the infinitesimal form of the refraction law is $dK_\perp = K_\parallel d\alpha$ where $K_\perp$ is understood as the function of $K_\parallel$ given by equation (2). Taken in conjunction with the equations $dr = v_\perp dt$, $r d\alpha = v_\parallel dt$ which describe the motion of the centre of the wave packet, with $\mathbf{v}$

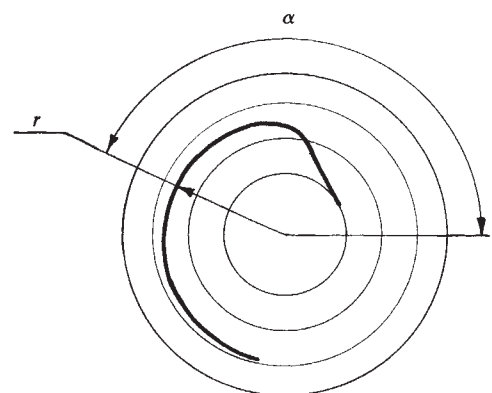


Fig. 2 The photon trajectory in a constant concentric magnetic field (the solid line). The circles denote the lines of force.

being the group velocity

$$\begin{aligned} \nu_{\perp} &= \frac{d\omega}{dK_{\perp}} = -\frac{c^2}{\omega} \frac{df}{dK_{\perp}^2} K_{\perp}, \\ \nu_{\parallel} &= \frac{d\omega}{dK_{\parallel}} = \frac{c^2}{\omega} K_{\parallel} \end{aligned} \quad (4)$$

this leads to the law of motion of the γ -quanta

$$\begin{aligned} t &= \frac{\omega r(0) K_{\parallel}(0)}{c^2} \int_{K_{\perp}(0)}^{K_{\perp}} \frac{dx}{K_{\parallel}^3(x^2)} \\ r &= r(0) \frac{K_{\parallel}(0)}{K_{\parallel}(K_{\perp}^2)}, \\ \alpha &= \int_{K_{\perp}(0)}^{K_{\perp}} \frac{dx}{K_{\parallel}(x^2)} \end{aligned} \quad (5)$$

Here the polar coordinates of the photon r, α and the time t are expressed as depending on the unique parameter $K_{\perp}$ which gives the corresponding position of the photon on the dispersion curve. The function $K_{\parallel}(x^2)$ is equation (2) using x^2 in place of $K_{\perp}^2$.

If the traditional dispersion law $K_{\parallel}^2 = \omega^2/c^2 - K_{\perp}^2$ is substituted into equations (5) the latter describe the photon propagating along a straight path with the constant speed c . If we approximate the dispersion law (2) by the broken line: $f(K_{\perp}^2) = -K_{\perp}^2$ for $K_{\perp}^2 < 4m^2c^2/\hbar^2$ and $f(K_{\perp}^2) = -4m^2c^2/\hbar^2$ for $K_{\perp}^2 > 4m^2c^2/\hbar^2$ we find that the photon moves directly until it gains the pitch angle ψ_1 (which happens at $r(t_1) = r_1 \equiv r_0[\omega^2/c^2 - K_{\perp}^2(0)]^{1/2}[\omega^2/c^2 - (4m^2c^2)/\hbar^2]^{-1/2}$), it then bends abruptly and propagates along the magnetic field (as now $\nu_{\perp} = 0$) with the constant angular velocity $(d\alpha/dt) = (c^2/\omega r_1)(\omega^2/c^2 - (4m^2c^2)/\hbar^2)^{1/2}$. For the very energetic γ -quantum $\hbar\omega \gg 4mc^2$, the group velocity $\nu_{\parallel} = c$ and $(d\alpha/dt) = (c/r_1)$. With the more exact function $f(K_{\perp}^2)$ (3) used in equation (5) the bending is less steep (see Fig. 2), the centre of the packet travels along a spiral which approaches asymptotically (from inside) the limiting circle, whose radius is

$$r(\infty) = r(0) \frac{K_{\parallel}(0)}{K_{\parallel}(\infty)} \equiv r_1$$

We call this phenomenon the capturing of γ -quantum by magnetic field.

Previously, we have been dealing with photons of only one polarization mode—that which has its electric field lying in the plane containing the magnetic field $\mathbf{B}$ and the direction of propagation $\mathbf{K}$. The other mode whose electric field is orthogonal to this plane, has its first virtual pair creation threshold at

$$\frac{\omega^2}{c^2} - K_{\parallel}^2 = \frac{2m^2c^2}{\hbar^2} + \frac{2eB}{c\hbar} + \frac{2mc}{\hbar} \left(\frac{m^2c^2}{\hbar^2} + \frac{2eB}{\hbar c} \right)^{1/2}$$

and not at threshold (1), because the two particles created by the photon of this mode cannot both be in the ground state—one of them must occupy the first excited Landau level⁵. This level being unstable, the threshold level also acquires some width. However, this does not affect the above conclusions about capture. As for the pair creation it does occur but only through tunnelling and is suppressed to this extent.

Consider now the restrictions on the magnetic field necessary for the above treatment. As already noted, the direction of the group velocity changes abruptly in the bending point if one uses the broken-line approximation for the dispersion curve. It follows from equations (2), (3) and (5) that the time t during

which this change occurs is about

$$r \sim \left(\frac{\alpha B}{B_{cr}} \right)^{2/3} \frac{mc^2}{\hbar\omega} \frac{r}{c} \exp \left(-\frac{4B_{cr}}{3B} \right)$$

The geometrical optics used above is justified if $rc \gg \lambda$, where λ is the photon wavelength. This is only if the magnetic field exceeds $\sim 0.1B_{cr}$. Other restrictions on the magnitude of the magnetic field are supplied by the following consideration. The derivation of dispersion law (2) was based on calculations which formally treated the magnetic field as external, that is, one which does not undergo any recoil. This approximation should be subjected to an *a posteriori* verification. This reduces to checking whether a given magnetic field can indeed provide the momentum transfer necessary to make the photon follow the law of motion (5). The corresponding analysis indicates that approximately the same condition as above is sufficient to provide the capturing, whereas a lesser field is required to keep the photon on the trajectory along the field once it has been captured.

We now discuss the consequences of the capturing of the γ -quanta in the magnetospheres of pulsars. According to Sturrock's model¹ of pulsars there is a gap near their magnetic poles wherein the component of the electric field $E_{\parallel}$ parallel to $\mathbf{B}$ is nonzero and equal to $(\Omega h_{max}/c)B_p \approx (\Omega R/c)^{3/2}B_p$, where $\Omega = 2\pi/P$ is the angular velocity of the pulsar, B_p is the magnetic field at its pole, R is the radius of the neutron star and h_{max} the length of the gap equal to $h_{max} = (\Omega R/c)^{1/2}R$. Using this model one could explain the energy yield of the system: the pulsar NP0532-Crab Nebula^{1,9}. A quantitative theory was also proposed which explains rather well the radiation spectrum of NP0532 and Crab Nebula^{10,11}. However, the length of the gap² cannot in fact exceed the free path l_{γ} , which the γ -quantum of the curvature irradiation passes before converting into the e^+e^- -pair, as the pairs created screen $E_{\parallel}$ and the length of the gap decreases to the value l_{γ} . For old (slowly rotating $P = 1$ s) pulsars, $l_{\gamma} \sim h_{max} \sim 10^4$ cm, that is the pair creation does not affect the gap in this case. For fast rotating pulsars the path l_{γ} , when evaluated using the assumption of the straight-line propagation of the photons, is essentially $< h_{max}$ for most of the γ -quanta of the curvature irradiation. For example, for NP0532 ($P = 0.033$ s) l_{γ} is less than h_{max} by ~ 1.5 orders. Consequently, in the framework of the existing theory of pulsars one cannot explain either the luminosity of Crab Nebula or that of the pulsar NP0532. The analogous difficulty is encountered, although to a lesser extent, with the pulsar PSR0833-45 in the remnant of Vela.

The effect considered here may overcome this difficulty. At $B \geq 0.1B_{cr}$ the γ -quanta of curvature radiation generated by the primary particles are captured by the magnetic field and leave the gap region without having created pairs at the ratio sufficient for its screening. Later the γ -quanta leave the domain of strong field. As the field becomes insufficient to keep them in its orbit we go beyond the range in which the above consideration is valid. One may assume, however, that the γ -quanta are released and may create pairs; this would occur at the distance of several radii of the neutron star.

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New evidence for degassing processes during explosive eruptions

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Measurements of pore size distribution in five samples of pumice, formed during explosive eruptions of plinian type, show three distinct sizes of vesicle. The characteristics of the three pore size populations are similar in the samples although they were collected from plinian deposits of five different volcanoes. We interpret the pore size populations as representing the degassing of silicic magma on three very different time scales. The coarse population ($>60\text{ }\mu\text{m}$) represents slow vesiculation within the magma chamber over periods of weeks, months or years at small levels of gas supersaturation. The medium-sized population ($50\text{--}5\text{ }\mu\text{m}$) represents rapid vesiculation of magma on ascent to the surface during the plinian eruption over periods of minutes to hours. The fine population ($5\text{--}0.5\text{ }\mu\text{m}$) represents bubbles formed during explosive disruption of magma and rapid ejection into the atmosphere, involving short time scales (a few seconds), but large decompression rates.

The pore size distribution of five individual samples of pumice collected from plinian deposits was measured using mercury porosimetry^{1,2} (a detailed description of the technique is given in ref. 2). The porosimetry method involves the forcing of mercury into a porous material under increasing pressure. The technique is based on the principle that when mercury is forced under pressure into a pore of characteristic dimension r and length l , an amount of work W is required which is proportional to the increased surface area exposed by the mercury at the pore wall. Theory¹ shows that the pressure P is related to the pore size, the surface tension of mercury γ and contact angle θ as follows:

$$P = (2\gamma \cos \theta)/r$$

The method allows the determination of pore size distribution from 300 to $0.003\text{ }\mu\text{m}$, surface area per unit weight of sample for pores greater than any specified value, and an approximate estimate of density. The method strictly measures the dimensions of entrances to pores rather than the internal dimensions. Duplicate runs on the same material using this method typically show a standard deviation of mean pore size of only 2–3%. The actual shapes and dimensions of pores have been examined by scanning electron microscopy (SEM) for comparison. The present results are preliminary but are the first measurements of the detailed pore size characteristics of volcanic ejecta.

The five samples studied were collected from the 18 May plinian deposit of the 1980 eruption of Mount St Helens (STH15), the Avellino plinian deposit of Vesuvius (IT540), the 1975 plinian deposit of Askja volcano, Iceland (AS10), the plinian deposit of the Tierra Blanca tephra, Lago de Ilopango, El Salvador (TBJ) and the plinian deposit of the Bronze Age Minoan eruption of Santorini, Greece (MIN). Table 1 lists some relevant properties of the pumice samples and results of density and surface area determinations.

Figure 1 shows differential pore volume plotted against pore diameter. The most striking feature of the data is that three discrete ranges of pore size are observed in all five samples despite the different localities. The Vesuvius sample shows the same pattern although the glass composition is trachytic whereas the other four samples are rhyolitic. The relative volumetric proportions of the three populations differ from sample to sample. For example, the finest-size population is dominant in the Vesuvius and Tierra Blanca samples, but is subsidiary in the Minoan sample. However, measurements on two other samples of Askja pumice clasts show considerable variation in the am-

Table 1 Properties of pumice samples and results of density and surface area determinations

Sample	Locality	Composition	Density (g cm^{-3})	Surface area ($\text{cm}^2 \text{g}^{-1}$)
STH15	3 km east of Mount St Helens	Dacite with rhyolite glass	0.91	8.47×10^4
MIN	Thera Quarry, Santorini	Rhyodacite with rhyolite glass	0.81	0.79×10^4
IT540	Ottaviano Village, Vesuvius	Trachyte	0.62	8.04×10^4
TBJ	Lago de Ilopango, Nicaragua	Rhyolite	1.03	0.54×10^4
AS10	15 km east of Oskjuvatn Caldera, Askja	Rhyolite	0.69	5.57×10^4

The measurements of surface area are for all pores down to a nominal diameter of $0.003\text{ }\mu\text{m}$.

plitude of the three peaks. This implies that many more analyses would be required to demonstrate significant differences in the overall size population distribution of the five pumice deposits. From the distributions in Fig. 1 we separated each pore size population and plotted the data on a graph of log pore diameter against cumulative volume per cent. The range of pore sizes in each population, as measured by the diameters at the 16th, 50th and 84th percentiles on the cumulative plot, is strikingly similar

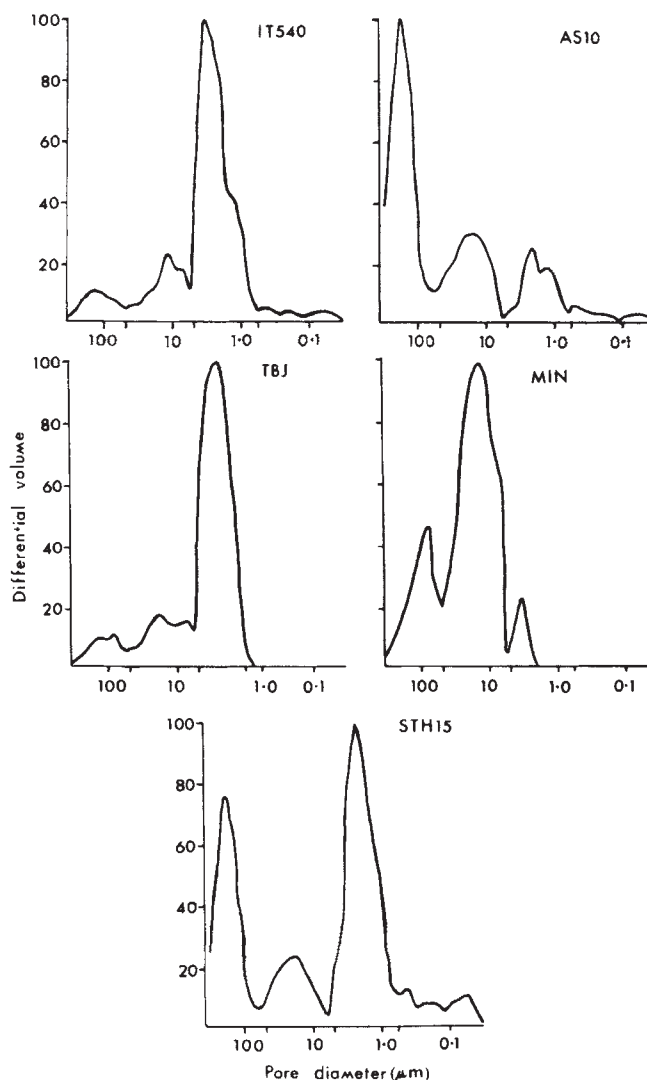


Fig. 1 Plots of differential volume against pore diameter for five pumice samples.

for the five samples. Table 2 lists the median pore diameter, pore size range and pore volume per gramme of sample for each population. In three samples (IT540, STH15 and AS10) significant proportions of pore sizes are observed down to $0.02\text{ }\mu\text{m}$ (Fig. 1) whereas in samples MIN and TBJ there are no significant proportions of pore sizes beneath $1.0\text{ }\mu\text{m}$. The porosimetry method only measures pores $<300\text{ }\mu\text{m}$ across. Much larger vesicles are observed in all our samples. Consequently the coarsest population cannot be completely determined.

Examination of the pumice samples by SEM establishes that the pore dimensions determined by porosimetry are representative of the true sizes of vesicles. Furthermore the different size populations of vesicles are also apparent. Figure 2 shows micrographs of sample IT540. Figure 2a shows a group of vesicles in the approximate size range $10\text{--}20\text{ }\mu\text{m}$ surrounded by areas characterized by much smaller vesicles. Figure 2b illustrates an area of the smaller-sized vesicles which are typically in the size range $1\text{--}4\text{ }\mu\text{m}$. The size ranges of the two groups of vesicles observed with the SEM are comparable with the middle-sized and fine-sized pore size populations (Table 2).

The three populations of pore sizes provide intriguing new evidence on the processes of degassing during major explosive volcanic eruptions. The occurrence of essentially the same pore size populations in samples from five different plinian deposits suggests a coherent pattern of degassing in this style of volcanic activity.

Models of explosive volcanic eruptions³ involving high viscosity magmas recognize three stages of vesiculation. Before an eruption magma oversaturated in volatile components will form bubbles within the chamber. Bubbles can reach substantial dimensions because the time scale for growth can be large (weeks, months, years, even centuries), although the over-saturation pressure causing gas to diffuse into the bubbles may be small⁴. We suggest that the population of coarse pore sizes represents vesiculation of gas within the chamber before eruption.

The second stage of vesiculation occurs as magma ascends to the surface just before and during the eruption. Plinian eruptions typically last a few hours to a couple of days with discharge rates of magma in the range $10^3\text{--}10^5\text{ m}^3\text{ s}^{-1}$ [ref. 3]. During this period magma is transported from depths of a few kilometres to near the surface where explosive disruption occurs. Models of this process^{3,4} suggest that explosive disruption occurs at pressures of a few tens of bars. Thus the magma decompresses from a few kilobars to a few tens of bars in typical periods of many minutes to a few hours. We propose that the medium-sized

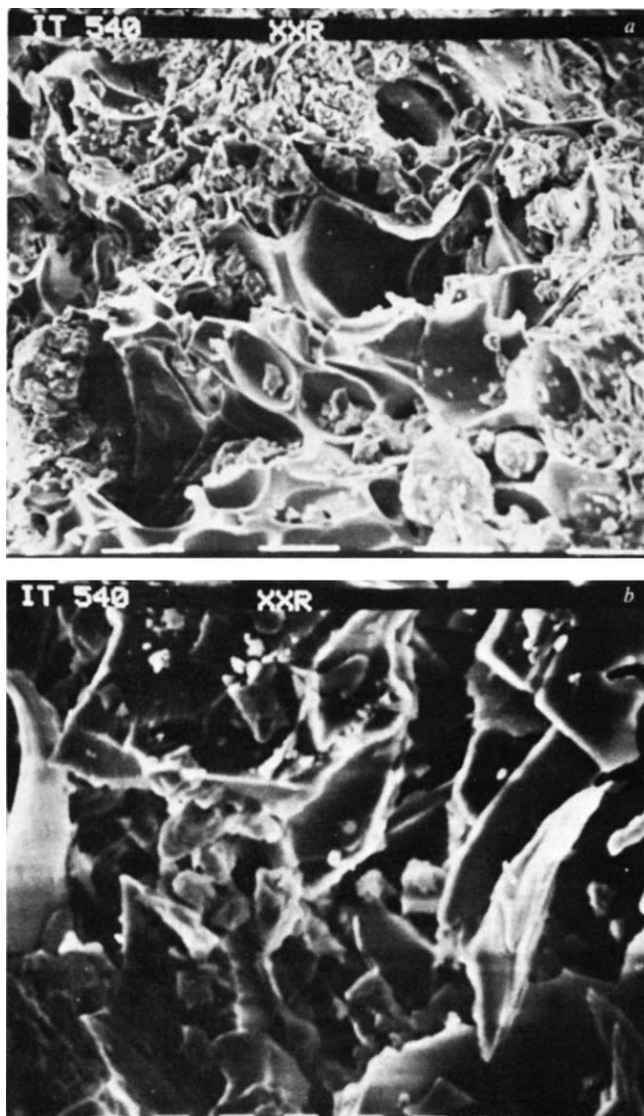


Fig. 2 Scanning electron micrographs of Avellino pumice sample (IT540). *a* Shows a group of large vesicles in the centre which correspond to the middle-sized pore population and areas of small vesicles which correspond to the fine-sized pore population. Scale bars, $10\text{ }\mu\text{m}$. *b* Shows an area of small vesicles which are prominent in the upper left-hand part of *a*. Scale bars, $1\text{ }\mu\text{m}$.

Table 2 Data on three pore size populations

Pore population	Sample	Median pore diameter (μm)	Pore volume ($\text{cm}^3\text{ g}^{-1}$)	Pore size range (μm)
Coarse	STH15	166	0.11	234–110
	MIN	98	0.14	173– 60
	IT540	119	0.07	202– 46
	TBJ	117	0.04	202– 71
	AS10	174	0.38	245–119
Medium	STH15	20.1	0.09	37.0–10.5
	MIN	14.4	0.60	28.1– 7.8
	IT540	12.3	0.21	27.9– 6.6
	TBJ	13.8	0.11	28.8– 6.3
	AS10	20.1	0.30	34.7–10.2
Fine	STH15	2.04	0.37	3.05–1.10
	MIN	3.80	0.05	4.90–3.02
	IT540	2.69	0.77	4.17–1.45
	TBJ	2.88	0.40	3.98–1.80
	AS10	1.91	0.25	2.91–1.17

The pore size range shows the 16th and 84th percentiles of each log normal distribution on a plot of cumulative volume versus pore diameter (see Fig. 1). The pore volume values are approximate estimates of the volume of each population group per gramme of sample.

population of pores are nucleated at this stage. We note that although decompression is rapid in this period, the loss of gas (assumed to be largely H_2O) from silicic magma causes a sharp increase in viscosity. For viscosities $>10^8$ poise bubble growth will be significantly retarded⁴. During this second stage the bubbles that had formed in the chamber will continue to grow by diffusion and decompression resulting in the clear separation of the two generations of bubbles observed in Fig. 1.

The final stage of vesiculation occurs during explosive disruption of the magma into the atmosphere. Velocities of the order $300\text{--}600\text{ m s}^{-1}$ and decompressions of the order of a few tens of bars are attained during plinian eruptions³. Rapid quenching of disrupted magma (pumice and ash) occurs as cold atmospheric air is mixed turbulently into the explosion column. Time for bubble nucleation and growth is restricted to a few seconds. However, we propose that the large pressure drop nucleates a large number of small bubbles and that the population of fine pores forms at this stage.

The data reveal an unexpected and distinctive pattern of three pore size populations in plinian pumice clasts. The three periods of vesiculation proposed to explain these populations seem to be the most obvious explanation. However, much further work is required to confirm this hypothesis. If our interpretations of the

data are correct, the degassing history of magma can be traced back into the chamber. We suggest that studies of pore sizes and surface area in volcanic ejecta may prove a fruitful way of studying the processes of volcanism. Studies of ejecta from different types of volcanic activity could provide new insights into volcanic mechanisms.

Measurements of pore size distribution were made on the Micromeritics Mercury Penetration Porosimeter Model MIC 9200 at Coulter Electronics Laboratory, UK. We thank R. Wenham for advice on the method and its interpretation. This research was carried out under NERC grant 6234.

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Ice core sample measurements give atmospheric CO₂ content during the past 40,000 yr

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Recent measurements^{1,2} on ice samples from Camp Century (Greenland, 77°10' N, 61°08' W), Byrd Station (Antarctica, 80°01' S, 110°31' W) and Dome C (74°40' S, 125°10' E) suggest that during the late part of the last glaciation the atmospheric CO₂ concentration was significantly lower than during the Holocene. Further investigation of this natural increase of the atmospheric CO₂ concentration in the past should aid our understanding of the climatic implications of the man-made CO₂ increase since the beginning of industrialization³. Here we report new and precise measurements of the CO₂ concentration of the air occluded in bubbles of ice samples from Camp Century and Byrd Station, using a new dry extraction technique. The extracted gases were analysed with an IR-laser spectrometer (IRLS). Samples from 22 different depths were analysed from each core. The samples are distributed over a depth interval corresponding approximately to the past 40,000 yr. In addition results for ice samples from selected depth horizons from a colder region (North Central, Greenland 74°37' N, 39°36' W) and from a warmer region (Dye-3, Greenland 65°11' N, 43°50' W) are given. Based on these results we estimate the trend of the atmospheric CO₂ concentration during the past 40,000 yr.

Samples of ~1 g were cut from the ice core with a band saw. The sample was placed into a cooled (–20°C) container between two needle matrices. The system was then evacuated and the ice cube crushed by pressing the needle matrices against each other. The gas originally trapped in the bubbles of the ice escaped and flowed into an absorption cell, where the CO₂ concentration was determined using an IRLS. The crushing process was very quick and the absorption peak was measured repeatedly for a few minutes. The fast extraction and the continuous monitoring of the CO₂ concentration of the escaped gas allowed us to check whether the measurement was affected, for example, by a small leak or CO₂ contamination from the surface of the container (R. Z., A. N. and H. O., in preparation). The precision of the measurements was 1.5%. Between two and eight samples were measured at each depth.

To determine the total inorganic carbon content, ice samples of 300 g were melted in an evacuated glass container and the gases extracted for 7 h. The samples were analysed with a gas

chromatograph⁴. If the melt water was alkaline, CO₂-free phosphoric acid was added to complete the extraction of CO₂. The total inorganic carbon content consisted of the CO₂ in the bubbles, the CO₂ dissolved in the ice matrix, the carbonates from dust incorporated in the ice and carbonates from the surface of the ice. Both the IRLS and the gas chromatograph were calibrated with three standard gases prepared by the Scripps Institution of Oceanography⁵.

Figure 1 shows the measured CO₂ concentrations plotted against depth, together with a simplified ¹⁸O/¹⁶O ratio curve derived from the detailed curve given by Johnsen *et al.*⁶. The originally recovered Camp Century and Byrd Station cores are generally of poor quality between the 400 and 1,200 m depths and the ice is heavily fractured. In these depth intervals the measured CO₂ values exhibit large scattering. This might be due to contamination by drilling fluid, a mixture of diesel fuel (88%) and trichlorethylene (12%), penetrating through small cracks into the ice. In these fractured ice zones, we measured several samples at each sample depth. Its small size meant that the sample was probably uncontaminated and we conclude that the lowest CO₂ values best represent the CO₂ concentrations of the originally trapped air of these depth intervals. In the larger samples (300 g) contamination is almost inevitable and the measured CO₂ concentrations tend to be higher than the air originally occluded in the ice. This is one reason for the high CO₂ values obtained during the climatic optimum given by Berner *et al.*¹ for the Camp Century core.

The CO₂ concentrations measured on 22 samples spaced over 11 cm from the 103-m depth, from North Central (Greenland), a colder location than Byrd Station and Camp Century, have a mean value of 271 p.p.m. The standard deviation of the measurements is only 9 p.p.m. This depth horizon corresponds to an age of ~680 yr. The gas enclosure occurs between 60 and 80 m corresponding to an age interval of 350–500 yr.

Figure 2 shows the CO₂ concentration variations measured on a 13-cm long ice core segment drilled in 1979 at Dye 3 (Greenland)—a location that is subject to frequent summer melt at the surface. Figure 2 thus shows the increased CO₂ concentrations that are usually associated with a melt feature.

Cold ice which forms by diagenetic processes and sintering of cold dry snow contains samples of atmospheric air in the bubbles. (This has also been shown for N₂, O₂ and Ar (ref. 7).) The results of the samples from North Central show that reproducible values are obtained for the CO₂ content using our improved extraction technique.

Due to atmospheric mixing, the CO₂ concentration in the Northern and the Southern Hemispheres should not differ by more than a few p.p.m. (ref. 8). For corresponding times one would expect the same CO₂ concentration in the bubbles in very cold ice from Antarctica and Greenland. Indeed, the CO₂ results of Camp Century and Byrd Station agree qualitatively. In both cores the decrease of the CO₂ content corresponds to the δ¹⁸O decrease which marks the transition from the Wisconsin to the Holocene.

However, there are differences between the Byrd Station and the Camp Century values which exceed the analytical errors. The higher CO₂ values in the Camp Century core during the Holocene may be due to the influence of melt water during the ice formation. In fact many melt layers have been observed in the Camp Century core (C. Langway and H. Clausen, personal communication). If meltwater is involved during the ice formation, the CO₂ concentration in the bubbles increases as shown by the results from the Dye 3 sample (Fig. 2). Although a detailed stratigraphical examination for the melt features was not made in the Byrd ice core the overall melt in this core is substantially less than recorded in the Camp Century core (C. Langway, personal communication). Therefore, for the Byrd core this effect should be negligible. At the end of last glaciation, when the CO₂ concentration was at a minimum, the Camp Century CO₂ concentration in the bubbles was slightly lower than those from the Byrd core. During this time the impurity content in the Camp Century core is an order of magnitude higher than in the

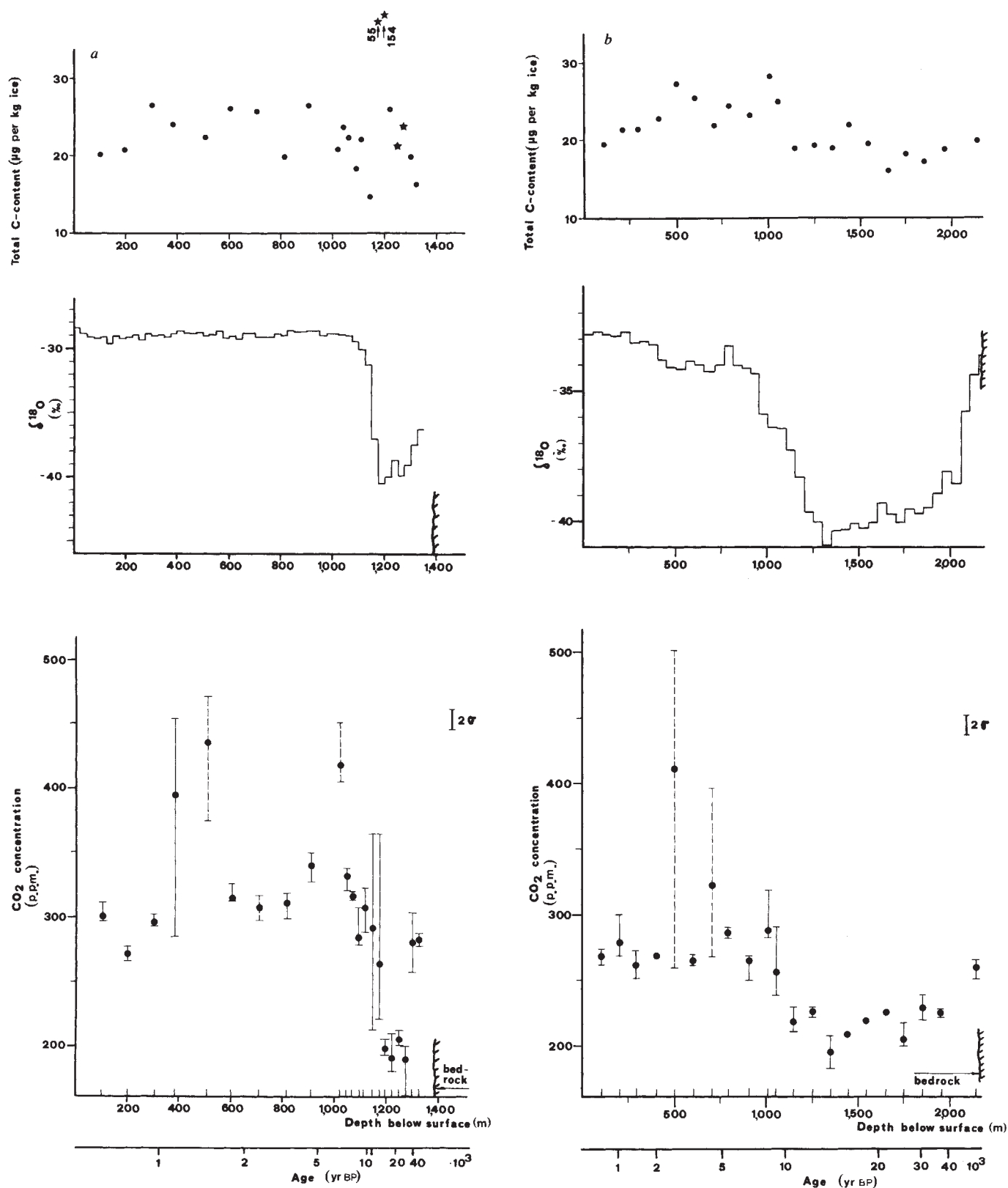


Fig. 1 CO_2 concentrations in the bubbles and total carbonate content of: a, the Camp Century core; b, the Byrd core. The CO_2 concentrations are presented with the lowest, highest and median values for each depth. The dashed lines indicate depths where drill fluid was observed in the large samples. All samples marked by a star were alkaline and acid was added during the wet extraction.

Holocene⁹ and the melted ice samples are alkaline. At present, we cannot rule out the possibility that the alkaline ice matrix absorbs a minor fraction of the CO₂ in the bubbles. Such a mechanism would explain the slightly lower Camp Century values for this period. While the carbonate content is an order of magnitude higher in Camp Century ice at the end of last glaciation than during the Holocene (Fig. 1), it remains almost unchanged in the Byrd core. The qualitative agreement between the two CO₂ concentration time series rules out the possibility of large interaction between CO₂ in the bubbles and the carbonates in the ice.

Note that the oldest samples in the Byrd Station and Camp Century cores (near the bottom of the ice sheets) again exhibit higher CO₂ values which are of the same magnitude as the Holocene values.

A generalized estimate of the history of atmospheric CO₂ concentration is shown in Fig. 3. The age of the Camp Century ice was calculated according to the method of Hammer *et al.*¹⁰. Several depth-age relationships for the Byrd core are given by Johnsen *et al.*⁶. We selected the time scale for Byrd Station, where the $\delta^{18}\text{O}$ decrease from the Holocene to the Glaciation coincides with the $\delta^{18}\text{O}$ decrease in the Camp Century core, that is, we assume the climatic events to be synchronous in both hemispheres. Due to the low data density and the uncertainties in the age calculations the estimate of the atmospheric CO₂ range in the oldest sections of the ice cores are more difficult to ascertain.

The increase of the atmospheric CO₂ content at the transition from the Ice Age to the Holocene and its climatic implications has to be discussed with regard to the global carbon system. The temperature change of the sea surface water and the change of ocean volume at this transition accounts for only a 5% increase of atmospheric CO₂ concentration¹¹. Both cannot explain the measured CO₂ increase of a factor 1.2–1.4 between the two time periods. The atmospheric CO₂ level depends on the carbonate chemistry of the surface ocean water, especially on the concentration of dissolved inorganic carbon (DIC) which in turn is controlled by biological productivity. Biological activity is limited by the amount of phosphorus available. Broecker links

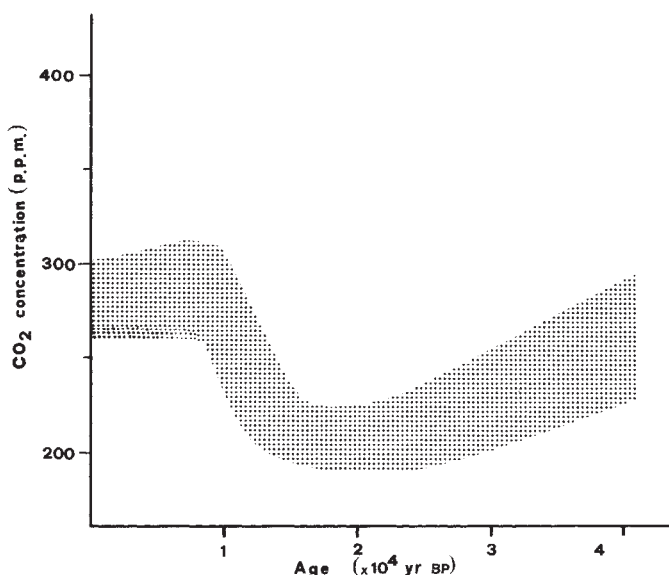


Fig. 3 Estimate range of atmospheric CO₂ during the past 40,000 yr.

the atmospheric CO₂ increase at the Glacial/Holocene transition with a change in the phosphorus available¹². He considers the following sequence of events. At the transition a higher sedimentation rate of organic material decreases the amount of phosphorus in the seawater. This leads to less biological productivity and therefore the difference in DIC between surface water and deep water decreases. This leads to a higher concentration of DIC in the surface layer and a higher atmospheric CO₂ concentration. The difference in DIC concentration between surface water and deep water is accompanied by a difference in $\delta^{13}\text{C}$ of the DIC. Simultaneous $\delta^{13}\text{C}$ measurements on foraminifera living at the surface and at the sea floor do indicate a higher vertical $\delta^{13}\text{C}$ gradient during the Glacial than during the Holocene, which agrees with Broecker's theory. The measurements of $\delta^{13}\text{C}$ values in the CO₂ gas extracted from ice samples could help us to understand the mechanisms which lead to the atmospheric CO₂ variations during climatic change.

Because CO₂ absorbs IR radiation, it is an important gas in the global radiation budget. Climatic models predict a global warming of between 1.5 and 4 °C for a doubling of CO₂ concentration in the atmosphere¹³. The relationship between the global temperature change and the CO₂ concentration change is approximately logarithmic¹⁴. A change in the atmospheric CO₂ concentration of 30% would lead to a global warming of $0.9 \pm 0.5^\circ\text{C}$. It has been reported that the global warming at the Glacial to Holocene transition was at least 2.3 °C (ref. 15). It therefore seems that the CO₂ increase alone was not large enough to explain all this global warming. Considering part of the temperature increase is due to an albedo change by a diminished ice or snow covered surface, the increased CO₂ level in the atmosphere contributes significantly to the global warming at the transition from the last glaciation to the Holocene. In this context it would be interesting to know whether the CO₂ increase preceded or followed the temperature change. Our data suggest that the time lag between the CO₂ increase and the climatic change cannot have been more than $\pm 2,000$ yr.

More accurate estimates of the atmospheric CO₂ history will be possible when samples from very cold locations, such as the South Pole, become available.

We thank Drs C.C. Langway, B.L. Hansen, H. Clausen and Professor W. Dansgaard who envisaged the potential of ice cores for studies of this kind and made them available for the present investigation, also Dr U. Siegenthaler for valuable discussions. This work was supported by the Swiss NSF the

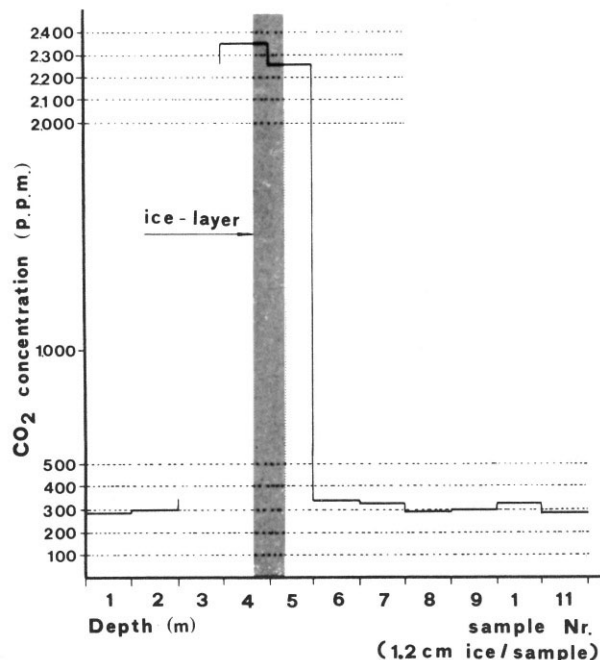


Fig. 2 CO₂ concentration in the bubbles of a 13-cm piece at a depth of 124 m of the Dye 3 core (southern Greenland).

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Steroid hydrocarbons and the thermal history of sediments

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Assessment of the extents to which certain organic chemical reactions have occurred in sedimentary rocks with increasing burial depth and the associated temperature rise, can distinguish differences in the extent of their thermal maturation^{1–10}. Heating experiments with sediments have suggested that these different reaction types have different kinetic constants¹¹. Therefore, a measurement of the extent to which one reaction type has occurred might be expected to correspond to different values of a measurement based on a different reaction type, depending, for example, on the average sedimentary heating rate ($^{\circ}\text{C Myr}^{-1}$)¹² of the sample. We use here two reaction types to investigate this hypothesis: (1) configurational isomerization in steroid alkanes, represented by the conversion of the biologically inherited configuration, $5\alpha(\text{H})$, $14\alpha(\text{H})$, $17\alpha(\text{H})$, 20R , in a C_{29} sterane to an approximately equal mixture of itself and the corresponding 20S configuration formed in the sediment^{6,13}; and (2) apparent aromatization of two C_{29} monoaromatic steroid hydrocarbons (Fig. 2) assumed to be isomeric at C-5 (refs 14–21), based on retention time comparisons with synthesized C-27 analogues, to a presumed product, the C_{28} triaromatic steroid hydrocarbon^{9,14}. Comparison of the extents to which these two reactions have occurred in suites of sediment samples has allowed three basins [Pannonian Basin (Pliocene), Mahakam Delta (Miocene), Paris Basin (Toarcian)] with different thermal histories to be distinguished. Extension of the hypothesis to two other sedimentary sequences suggests a higher average heating rate for the Oligocene of the Zhanhua Depression (north-east China) than for the Cretaceous of the Wyoming Overthrust Belt.

The thermal history of a sediment, that is, the temperature versus time profile since deposition, is important for estimating the time at which the sediment was capable of generating petroleum²². A simple means of describing thermal history is the average heating rate, defined as the product of average sedimentation rate (km Myr^{-1}) and geothermal gradient ($^{\circ}\text{C km}^{-1}$)¹². Table 1 lists the rates (based on present depth/age) for three sample suites: (1) 12 Pliocene shales from a borehole in the Pannonian Basin in south-east Hungary²³; (2) three shales and six coals from boreholes around the Handil oilfield of the

Mahakam Delta, Kalimantan, Indonesia²⁴; and (3) 14 Toarcian shales sampled from outcrops and boreholes across the Paris Basin^{6,25}. Although different types of organic matter occur in the sequences^{23–25}, the present comparison is valid because the carbon skeletons of the products of the reactions are only formed by thermal maturation and not by biological processes. Where there has been a period of erosion [(2) and (3)] corresponding heating rate values are given in Table 1, which do not include the periods of erosion. In either case there are major differences in heating rates over the three sample suites.

All the samples contain complex mixtures of stereoisomers of steroid alkanes with both a non-rearranged and a rearranged carbon skeleton, covering a range of carbon numbers^{2,5,6,26}, and can be partly separated by combined gas chromatography–mass spectrometry (GC–MS)^{5,6}. The complexity of the mixture increases with increasing thermal maturation due to isomerization at various chiral centres in skeletons⁶. Using non-polar GC columns the ratio (%) of the unresolved mixture²⁶ of the 24R and 24S isomers of (20S)-24-ethyl- $5\alpha(\text{H})$, $14\alpha(\text{H})$, $17\alpha(\text{H})$ -cholestane to itself and the corresponding 20R mixture can be measured from the appropriate peak areas in GC–MS fragmentograms of the ion m/z 217 (refs 5, 6) (Fig. 1). The value of this measurement rises from zero in very immature sediments to a maximum of ~50–55% in mature sediments^{5,6}, that is the equilibrium constant is ~1, although it may vary slightly with temperature.

The samples also contain complex mixtures of monoaromatic and triaromatic steroid hydrocarbons. The latter are thought to derive from the monoaromatics as a result of thermal maturation^{9,14}. The ratio (%) of the C_{28} triaromatic component in Fig. 2 to itself and two presumed precursors, thought to be the 5α and $5\beta(\text{H})$ isomers of the C_{29} component in Fig. 2, can be measured from mass fragmentograms of the ions m/z 231 and 253 respectively⁹. The two C_{29} monoaromatic components are suggested to be the 5α and $5\beta(\text{H})$ isomers of the structure in Fig. 2, the assignment being based on 400 MHz ^1H NMR analysis of a 1:1 mixture (GC analysis) of two stereoisomeric ring C monoaromatics isolated from acidic treatment²⁷ of cholesta-3,5-diene. Comparison of chemical shifts with literature compounds^{14–19} in conjunction with decoupling and n.o.e. experiments indicates: (1) ring C is aromatic (confirming the m/z 253 base peak in the mass spectra¹⁵); (2) there is a methyl group at C-17, consistent with the structures in the triaromatic series^{9,14,16}, and at C-10; (3) the A/B ring junction is *trans* with the $10\beta(\text{CH}_3)$, $5\alpha(\text{H})$ stereochemistry; (4) the 1:1 mixture results from isomerism at C-17 or C-20; that the latter is the case is suggested both by the structures and distribution of the low-molecular weight triaromatics⁹ and by the method of preparation²⁷. The two isomers coeluted (GC–MS) on OV-1 and Carbowax 20M with two major C-27 monoaromatics in the aromatic fraction of Semécourt shale (Paris Basin, see Fig. 3a). The other two major C-27 monoaromatics in this fraction are inferred to be the $5\beta(\text{H})$, 20R and 20S isomers. Comparison of elution orders of the C-27 and C-29 monoaromatic components suggests that the two C-29 components used in the measurement are those in Fig. 2 [before completion of this work it came to our attention that certain monoaromatic hydrocarbons of this structural type had been assigned and found to occur in sedimentary rocks and petroleum (W. K. Seifert and J. M. Moldowan, personal communication). The above conclusions are also consistent with structural assignments reported

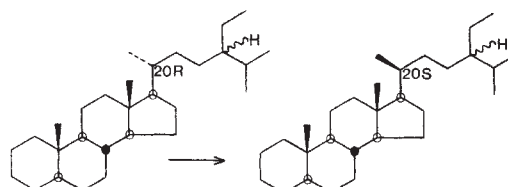


Fig. 1 Isomerization at C-20 of (24R+24S)–(20R)-24-ethyl- $5\alpha(\text{H})$, $14\alpha(\text{H})$, $17\alpha(\text{H})$ -cholestane with increasing thermal maturation.

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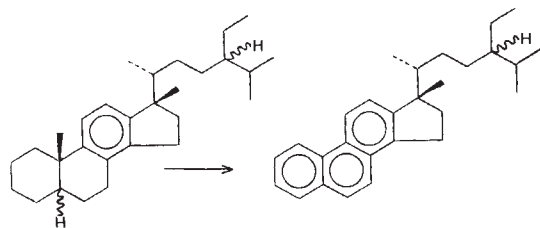


Fig. 2 Proposed aromatization of the $5\alpha(\text{H})$ and $5\beta(\text{H})$ isomers of (24R+24S)-(20R)-24-ethyl-17 β -methyl-18-norcholesta-8,11,13-triene to (24R+24S)-(20R)-24-ethyl-17 β -methyl-18,19-dinorcholesta-1,3,5(10),6,8,11,13-heptaene (numbering based on steroid skeleton).

recently^{20,21}]. Again the 24R and 24S isomers are not resolved on non-polar columns in the conditions used. The maximum value of the measurement is $\sim 100\%$ (ref. 11), and values as low as zero can occur in immature sediments⁹.

Figure 3a shows the isomerization measurement plotted against the aromatization measurement for the three sample suites. In the Paris Basin Toarcian shales, whose values and maximum burial depths have been reported previously^{6,9}, there is a steady increase, with some scatter, in both measurements with increasing burial depth such that the presumed end points of both reactions are approached in the two deepest shales. Heating an immature Toarcian shale at elevated temperatures in the laboratory resulted in aromatization values approaching the deepest shales, whereas only minor changes were observed in the extent of isomerization at C-20 in the steranes¹¹. It has been suggested that the rate of aromatization of the monoaromatic steroid hydrocarbons is more temperature dependent than the rate of isomerization of $5\alpha(\text{H})$, $14\alpha(\text{H})$, $17\alpha(\text{H})$ -steranes at C-20 (ref. 11).

Although the two reactions seem to have similar average rates at the lower temperatures ($<100^\circ\text{C}$) experienced by the Toarcian shales (Fig. 3a), comparison with the laboratory experiments (201, 257°C) suggests that the aromatization reaction occurs at a faster relative rate at higher temperatures. If the reactions follow pseudo first-order kinetics, then according to the Arrhenius equation, the rate constant for the aromatization of steroid hydrocarbons would appear to have higher values of activation energy and pre-exponential factor than the isomerization at C-20 in $5\alpha(\text{H})$, $14\alpha(\text{H})$, $17\alpha(\text{H})$ -steranes. ($\log_e k = \log_e k_0 - E/RT$, where k = reaction rate, k_0 = pre-exponential factor, E = activation energy, T = absolute temperature and R = gas constant).

This hypothesis has been examined by comparing the extents of the two reactions in the Toarcian shales with the sample suites from the Pannonian Basin and Mahakam Delta (Fig. 3a). The Pliocene of the Pannonian Basin has average heating rates at least 20 times higher than the Paris Basin Toarcian shales (Table 1). Details of aspects of the sedimentation and petroleum geochemistry of the Pannonian borehole have been reported elsewhere²³. The four shallowest samples (between 2,051 and 2,500m) show a trend (Fig. 3a) of enhanced aromatization

relative to isomerization, akin to that seen in the laboratory heating experiments¹¹; the aromatization (more temperature dependent) reaction is effectively complete by sample 4 (2,500m, $\sim 105^\circ\text{C}$ present temperature). At this depth the isomerization (less temperature dependent, and more time dependent) has reached a value of only 16% of the 20S isomer as a proportion of the C_{29} $5\alpha(\text{H})$, $14\alpha(\text{H})$, $17\alpha(\text{H})$ -steranes (Fig. 3a). High values (~ 50 – 55%) in the curve are not reached until a further depth increase of $\sim 800\text{m}$ (3,328m, sample 15, 137°C). The coal and shale samples from the Mahakam Delta derive from boreholes around the Handil oilfield. Detailed information about the geology and geochemistry is given elsewhere^{24,28}. The heating rate represents an intermediate situation between the Paris and Pannonian Basins (M. Vandenbroucke, personal communication). The path followed by the Mahakam Delta samples seems to reflect this intermediacy, although the Der sample from the Paris Basin has similar values to Mahakam samples 3, 9 and 10. The apparently anomalous behaviour of Der (and perhaps Colombotte) may relate to the fact that the Paris Basin samples derive from a wide geographical area⁶ and that Der (and Colombotte) may have experienced a period of higher sedimentation²⁹ not apparent from a study of the present record of sedimentation³⁰.

Average sedimentary heating rates can only reflect the overall thermal history of a sequence. It might be expected, however, that the molecular measurements could also reflect variation in the heating rate at a later stage. If the heating rate declines markedly, or even if cooling occurs, the decrease in the rate of the isomerization reaction (less temperature dependent) would be less than the decrease in the rate of the aromatization reaction (more temperature dependent). During such a period, a trend reflecting a high heating rate such as in the Pannonian Basin (Fig. 3a) would be displaced towards the Paris Basin trend. In the case of the Mahakam Delta suite, the samples have experienced heating rates of at least $30^\circ\text{C Myr}^{-1}$ (M. Vandenbroucke, personal communication) during the late Miocene, which are much greater than the average values for the Pannonian Pliocene. Since then, they declined such that they never exceeded 3°C Myr^{-1} during the late Pliocene and subsequent cooling of the samples has occurred during the past 3 Myr, because $\sim 300\text{m}$ has been eroded from the sequence studied (M. Vandenbroucke, personal communication). These events explain both the occurrence of samples of late Miocene age at the same depths as late Pliocene Pannonian samples, and the proximity of the Paris Basin and Mahakam Delta trends (Fig. 3a) which are not obvious from the heating rate differences (Table 1).

Figure 3b includes the two measurements plotted for (1) six Cretaceous shales from the Wyoming Overthrust Belt¹³; all but one are shallow (20–40m) core samples and derive from a sequence of increasing age within a single thrust slice. Calculation of average heating rates is difficult because of the structural complexity of the area³¹; (2) six Oligocene (Shahejie) cored shale samples from the Zhanhua Depression in north-east China, of varying burial depth and location³². Because the area

Table 1 Average heating rates for sample sequences

Sequence	Ref.	Age*(Myr)	Geothermal gradient ($^\circ\text{C km}^{-1}$)	Average sedimentation rate (m Myr^{-1})†	Average heating rate‡ ($^\circ\text{C Myr}^{-1}$)
Pannonian Basin	16	Pliocene (5–7)	38	360–420	14–16
Mahakam Delta	17	Miocene (12–14)	34	160–200 (250–290)§	6–8 (8.5–10)§
Paris Basin	6, 9, 25	Toarcian (180)	33	0–15 (5–15)§	0–0.5 (0.2–0.5)§
Overthrust Belt	13	Cretaceous (80–130)	NK	NK	–
Zhanhua Depression	32	Oligocene (25–40)	36	NK	–

NK, not known.

* Age of samples for which changes in one or both of the two molecular measurements are seen.

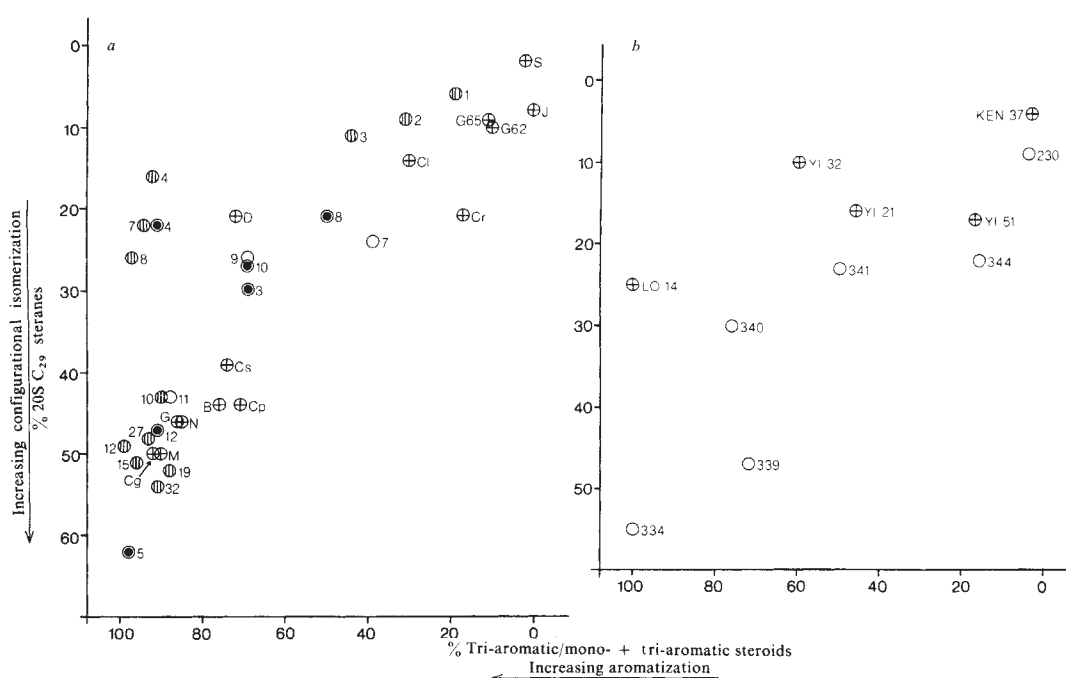
† Depth/age.

‡ Assuming constant geothermal gradient.

§ Excluding erosion phases.

|| M. Vandenbroucke, personal communication. Data for Handil field.

Fig. 3 Plots of the extent of isomerization at C-20 in C_{29} steranes (Fig. 1) against the extent of aromatization of C_{29} monoaromatic to C_{28} triaromatic steroid hydrocarbons (Fig. 2) for *a*, (maximum burial depths in brackets, where available): Toarcian shales of the Paris Basin ($\oplus$) where S, Semécourt (700m); J, Jouy (700m); G62, G6-2-2 (600m); G65, G6-5-6 (600m); Cl, Colombotte (500m); Cr, Creveney (500m); D, Der (1,280m); Cs, Cesarville (2,040m); Cp, Coupvray (2,100m); G, Grandville (2,100m); N, Nangis (2,130m); B, Belou (2,207m); M, Montmirail (2,433m); Cg, Courgivaux (2,460m). Miocene coals ($\bullet$) and shales ($\circ$) from the Mahakam Delta (Handil field except 3,4,5) where 7 (2,285m); 8 (2,283m); 9 (2,538m); 10 (2,537m); 11 (2,903m); 12 (2,905m). For samples 3, 4, 5 only T_{max} values³⁹ are available and are 430, 440 and 448 °C respectively (M. Vandenbroucke, personal communication). Pliocene shales and siltstones from a borehole in the Pannonian Basin ($\bullet$) where 1 (2,051m); 2 (2,291m); 3 (2,386m); 4 (2,500m); 7 (2,702m); 8 (2,759m); 10 (2,953m); 12 (3,098m); 15 (3,328m); 19 (3,548m); 27 (4,269m); 32 (4,888m). *b*, (Present depths) Cretaceous shales ($\circ$) from Wyoming Overthrust Belt (all shallow core samples (20-40m) except 334; present depth 2,760m). Oligocene (Shahejie) shales from the Zhanhua Depression ($\oplus$); present burial depths in brackets, Ken 37 (1,850-1,860m); YI 51 (2,339m); YI 21 (2,758m); YI 32 (2,899m); LO 14 (2,978m). The isomerization for LO 14 is an average of the C_{29} measurement and the corresponding measurement for the C_{27} skeleton, because there is a reworked component in the steranes with a very high relative abundance of C_{29} steranes³².



which was sampled has been affected by faults active till the Quaternary, and the samples are from separate boreholes of different location separated by up to 15 km, they presumably have some differences in their average heating rates, the range of which is difficult to estimate. Although the sample coverage is less extensive than in the other sequences (Fig. 3a), measurements suggest that the Overthrust Belt shales (Fig. 3b) have a similar average heating rate to the Paris Basin samples (Fig. 3a), whereas the Zhanhua Depression shales seem to have had a recent heating experience of a similar order to that which has affected the shallow Pannonian Pliocene samples. Such observations are consistent with available geological information for the two areas^{31,32}.

Comparison of proposed maximum temperatures for the Pannonian²³ and Paris^{33,34} Basins provides further evidence that the aromatization has the greater temperature dependence. For a given aromatization value the maximum temperature difference in samples from the two basins is of the order of 10 °C, whereas for a given isomerization value differences of up to 60 °C are apparent.

Confirmation and extension of the hypothesis requires: (1) determination of the appropriate activation energies and pre-exponential factors, both from laboratory heating experiments¹¹ (with consideration of the difficulties involved³⁵) and from integration with mathematical models for the evolution of sedimentary basins^{36,37}, which include temporal variations in sedimentation rate and heat flux. Preliminary studies of the latter type, using estimated kinetic constants for the reactions, suggest that the trends (Fig. 3) follow S-shaped curves (A. S. M. and D. P. McKenzie, unpublished results). (2) An examination of the effect which extreme differences in lithology could have on the measurements. In the present study, all the samples are shales or silt-stones except for the six coal samples from the Mahakam Delta in which little significant variation was observed between the coals and shales from comparable depths (Fig. 3a). (3) A search for other measurements which might preserve a record of earlier high heating rate events, although subsequent

lower heating rates and/or cooling result in a trend which suggests an overall low average heating rate (see Mahakam).

Care should be taken with the measurements: the mixtures are complex and overlap with other components can occur. Attention should be paid to peak shapes in various diagnostic fragmentograms, to examination of full mass spectra when required, and to the potential presence of migrated components.

This approach has not been extended to a study of crude oils, where migration and subsequent thermal effects in the reservoir (as opposed to those in the source rock) could affect the measurements. Preliminary studies^{13,32,38} suggest that the configuration at C-20 is not altered significantly by either process, although the proportion of triaromatic to monoaromatic steroid hydrocarbons can decrease in certain cases with increasing distance of secondary migration (unpublished results on Mahakam Delta oils).

If the hypothesis is correct there are other implications. For example, there is no reason why variation in the reflectance of vitrinite, commonly used to determine the onset of petroleum generation in sediments, should follow the same kinetic behaviour as the petroleum generation process. Reflectance values are different for the threshold of petroleum generation from different types of organic matter²², but the hypothesis suggests that there may also be differences within a single type of organic matter, depending on heating rate differences.

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Fine-scale isotopic heterogeneity in the sub-Atlantic mantle

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The isotopic composition of Sr and Nd in oceanic basalts reflects the character and chemical evolution of their source regions with respect to Rb/Sr and Sm/Nd. Combined studies of the Sr, Nd and Pb isotopic systems in mid-oceanic ridge basalts (MORB)^{1,2} have confirmed previously recognized heterogeneities on a relatively large scale for the oceanic mantle. These heterogeneities have been ascribed to both long- and short-lived mantle processes and to mixing between enriched and depleted mantle sources (for example, see refs 1–4). However, data for the mid-Atlantic ridge (MAR) remote from known hot spots are available for only 14 samples of which only 3 are basaltic glass, the most 'reliable' sample type. Thus, attempts to explain MORB mantle heterogeneities are founded on limited data and remain largely speculative. The extent of variation in isotopic

composition of 'normal MORB' over a limited area of the MAR is critical to any isotopic model for the oceanic mantle. Using high resolution seafloor mapping and sampling techniques, we can evaluate isotopic composition on a fine scale both within a ridge segment and across the fracture zones terminating that segment. We report here studies of a series of basaltic glass samples from the MAR intersection with the Kane Fracture Zone located at 22°–24° N (Fig. 1) which have implications for the dynamics of mantle melt segregation and the role of a major transform in mantle convection.

The Kane Fracture Zone is particularly interesting as it is one of the few areas of the MAR where the basalts are interpreted to represent 'normal MORB' and for which geochemical data indicate a relatively simple history of fractional crystallization and partial melting⁵. The basalts of the Kane Fracture Zone do not display geochemical complications such as crossing rare-earth element patterns and complex mixing relationships that have been identified for the MAR axis north of 30° N (refs 6, 7). In addition, phase relations and geochemical models⁵ indicate that, in many cases, the magma batches that erupted in the 22°–24° N region have evolved independently, and mixed only over a limited geographical area. This minimizes the likelihood that large magma chambers or extensive melt segregation and mixing in the upper mantle have homogenized the isotopic systems, and this in turn, enhances the possibility of exposing and resolving fine-scale mantle heterogeneity.

All analyses were performed on glass samples to minimize the problem of seawater contamination. After separation by hand picking, the glasses were washed in acetone and leached in 6.2 HCl. Sr and Nd were isolated by ion exchange following the techniques of Hart and Brooks⁸ and Richard *et al.*⁹ respectively. All samples were analysed on a modified, automated NBS 12-inch mass spectrometer at the Université de Montréal. Nd and Sm were analysed on a double Ta–Re filament arrangement, whilst all other elements were run on a single Ta filament. The data for the glass samples plus Sm, Nd and ¹⁴³Nd/¹⁴⁴Nd data for the rock standard BCR-1 and Nd oxide JMC-321 are given in Table 1. The sample identification conforms to the usual Woods Hole Oceanographic Institution's designation where G104-25-1 indicates sample number 1 from station 25 of RV Gilles Cruise 104.

When all the data from 22°–24° are considered, the compositions fall at the low ⁸⁷Sr/⁸⁶Sr and high ¹⁴³Nd/¹⁴⁴Nd end of the Sr–Nd correlation^{1,2,9–11} and extend the range for oceanic basalts to the most depleted values yet reported from the sea floor. Figure 2 shows that the fracture zone clearly marks a barrier to isotopic domains. The compositions of the samples from the ridge axis and from the fracture zone walls from the northern section are isotopically distinct from those of the southern section. The range in variation in ¹⁴³Nd/¹⁴⁴Nd isotopic composition exceeds that of ⁸⁷Sr/⁸⁶Sr, resulting in a vertical distribution across the oceanic trend similar to that observed for Iceland¹² and for the Marianas¹³.

The abundances of the most lithophile elements show

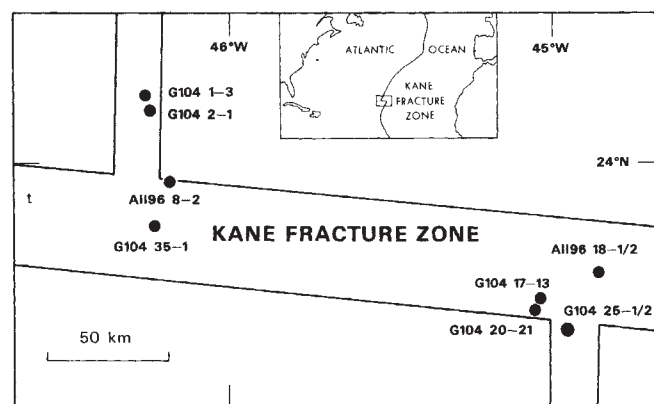


Fig. 1 Sample location map for the Kane Fracture Zone.

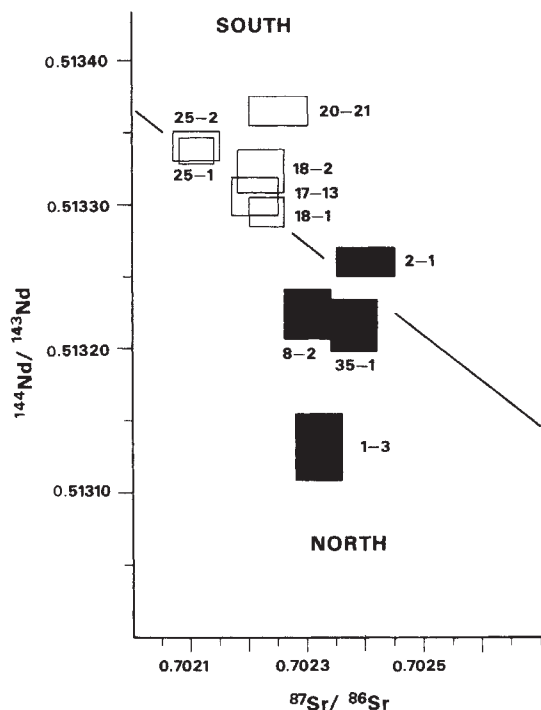


Fig. 2 $^{143}\text{Nd}/^{144}\text{Sr}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ data for the Kane Fracture Zone basalt glasses (open symbols, southern intersection; solid symbols, northern intersection).

considerable variation both within a ridge section and between the northern and southern sections. K/Rb varies from 1,250 to 1,820 in the southern ridge section relative to a variation of 680–760 in the northern section, whilst K/Ba values are of the order of 165 and 65 respectively. Both K/Rb and K/Ba exhibit an inverse correlation with $^{87}\text{Sr}/^{86}\text{Sr}$. Although there is some overlap between the northern and southern sections, the samples define a positive correlation on the Rb/Sr isochron

diagram (Fig. 3a) which if interpreted as an age corresponds to 805 Myr (with an $^{87}\text{Sr}/^{86}\text{Sr}$ $R_i = 0.70205$). In contrast, the samples have similar Sm/Nd in both data sets with the exception of sample G104-2-1 which has a higher ratio. This sample and others from this site are anomalous in having high Sr abundance relative to those samples from the northern section (Fig. 3c).

The most significant geochemical property of the glass samples is their extreme trace element depletion in the southern section (as manifest in K/Rb, K/Ba and Rb/Sr ratios), and the correlation of that depletion with isotopic depletion. Possible mechanisms by which these data can be explained include: (1) long-lived mantle heterogeneity developed during the evolution of the sub-oceanic mantle; (2) a recent mixing event involving an enriched component added to and mixed incompletely with the oceanic mantle (such as a plume source and lateral transfer away from the plume); (3) transfer of volatile elements from the lower to the upper mantle.

The geographical constraints, in which the samples from the northern section are enriched relative to those from the southern section are fundamental in appraising these models. For instance, we see no plausible mechanism by which a superficial (lithospheric) structure such as a transform fault could control the observed distribution in isotopic ratios that would arise during volatile transfer of variably enriched fluids from the lower to the upper mantle. Such geographical control requires either a lateral transfer of relatively enriched material in the upper mantle, or mantle heterogeneity developed before or during the initial opening of the Atlantic Ocean. The fracture zone is thus controlling or is controlled by the heterogeneity.

Two lines of isotopic evidence can be used to support a mixing model. First, samples from the northern section are more radiogenic than those from the southern section and may thus represent mixing of enriched and depleted material along a N–S axis. Second, there are correlations defined by the samples from the southern section (Fig. 3c, d), which on first examination could be interpreted as mixing lines. Bryan *et al.*⁵ cite trace element data that support magma mixing over limited regions for the southern section. However, data such as K/Rb, which confirm the more enriched character of the mantle in the northern section, are inconsistent with a simple mixing model. For instance, K/Rb for site 20 is higher than that in the more depleted samples of site 17. When the 1/Sr and 1/Nd diagrams

Table 1 Isotopic and trace element analysis (p.p.m.) of basaltic glasses

Sample	Lat. N	Long. W	$^{87}\text{Sr}/^{86}\text{Sr}$	$^{143}\text{Nd}/^{144}\text{Nd}$	K	Rb	Sr	Ba	Sm	Nd	K/Rb	K/Ba	Rb/Sr	Sm/Nd
G104 1-3	24°11.0'	46°16.9'	0.70242 ± 4*	0.513132 ± 23*	806	1.11	126.2		3.45	9.78	729	—	0.009	0.353
G104 2-1	24°09.4'	46°16.1'	0.70250 ± 5	0.513260 ± 19	1,233	1.77	140.5	19.81	3.47	7.58	696	62	0.013	0.458
AII96 8-2	24°00.7'	46°11.2'	0.70240 ± 4	0.513224 ± 17	—	—	—	—	—	—	—	—	—	—
G104 35-1	23°51.2'	46°13.4'	0.70248 ± 4	0.513216 ± 18	812	1.07	127.9	11.28	3.59	10.20	757	72	0.008	0.352
AII96 18-1	23°31.5'	44°49.1'	0.70233 ± 3	0.513295 ± 10	—	—	—	—	—	—	—	—	—	—
AII96 18-2	23°31.5'	44°49.1'	0.70232 ± 4	0.523323 ± 15	—	—	—	—	—	—	—	—	—	—
G104 17-13	23°37.2'	45°02.1'	0.70231 ± 4	0.513306 ± 13	1,509	1.21	138.5	—	8.68	25.61	1,249	—	0.009	0.339
G104 20-21	23°37.1'	45°02.3'	0.70235 ± 5	0.513365 ± 10	1,488	0.92	138.1	—	4.31	12.51	1,619	—	0.007	0.345
G104 25-1	23°32.0'	44°57.4'	0.70221 ± 4	0.513341 ± 10	1,401	0.60	139.5	6.41	4.93	13.78	1,735	162	0.004	0.357
G104 25-2	23°32.0'	44°57.4'	0.70221 ± 3	0.513338 ± 9	1,135	0.62	140.8	6.56	5.05	14.42	1,821	173	0.004	0.348
BCR-1			—	0.512679 ± 20	14,278	46.91	330.0	702	6.71	28.65	—	—	—	—
JMC321				0.511921 ± 12										
Total blank	(ng per g)				9	0.063	0.2	3	0.1	0.2				

* s.e. at 2σ.

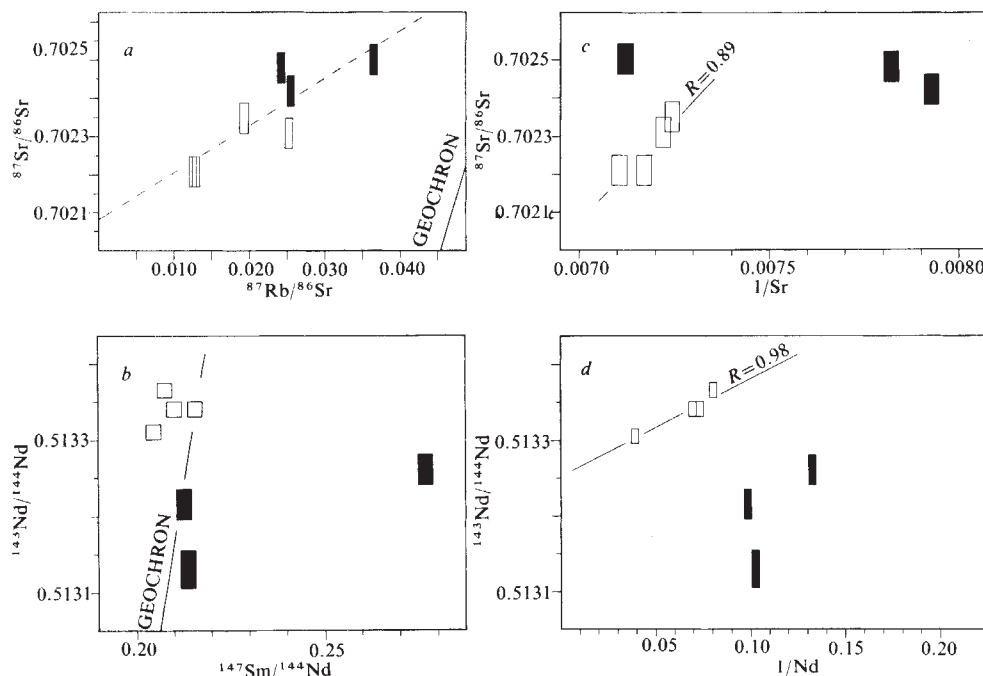


Fig. 3 *a*, Rb-Sr isochron diagram; *b*, Sm-Nd isochron diagram; *c*, $^{87}\text{Sr}/^{86}\text{Sr}$ against $1/\text{Sr}$; and *d*, $^{143}\text{Nd}/^{144}\text{Nd}$ against $1/\text{Nd}$ for the Kane Fracture Zone basalt glasses.

are compared they reveal similar inconsistencies. For example, one of the end members of the southern section on the $1/\text{Sr}$ diagram plots between the end members on the $1/\text{Nd}$ diagram. Although there is no positive confirmation of mixing based on the isotopic data, we cannot discount the possibility that on a small scale (that is between adjacent sites or within a single site; see ref. 5) mixing may have occurred.

The isochron diagrams (Fig. 3*a, b*) reveal that whilst the samples define a 'reasonable' age on the Rb/Sr isochron plot, they define ages older than Earth on the Sm/Nd diagram. This decoupling of parent-daughter isotopes in the Sm/Nd system is interpreted to be a result of recent petrogenetic processes involving garnet and clinopyroxene¹⁴. Despite these complications, which, for example, result in a vertical distribution on the Sm/Nd isochron plot, we distinguish three subsets on the Sr/Nd correlation diagram (Fig. 2) and the Rb/Sr isochron diagram (site 25; sites 1, 2, 8, 35 and sites 17, 18, 20). These subsets may reflect heterogeneities that developed during either a single event¹⁵ or an extended period of mantle differentiation¹⁶. However, the correlation of these subsets on the Rb/Sr isochron diagram implies that, even on a small scale, the mantle has remained heterogeneous over a considerable period.

Trace element data for the MAR between the Kane Fracture Zone and 37° N (ref. 17) indicate that major transforms such as the Hayes Fracture Zone delineate mantle regimes. No isotopic data are available for the MAR between the Kane Fracture Zone and the FAMOUS region (36° N) and we cannot evaluate isotopic heterogeneity across the Hayes transform. However, due to the lack of simple mixing relationships between the northern and southern sections of the MAR at the Kane Fracture Zone we speculate that, in some cases, a transform may act as a barrier to different mantle domains. Support for this model comes from $^{87}\text{Sr}/^{86}\text{Sr}$ data for the Gibbs Fracture Zone⁴ for which the values vary from ≈ 0.70285 in the northern section (60°–53° N) to 0.70225 in the southern section (53°–49° N). Furthermore, White and Schilling⁴ indicate a sharp increase in $^{87}\text{Sr}/^{86}\text{Sr}$ initial in the region 49°–43° N which supports our contention that lateral flow away from an enriched mantle source is limited geographically. Thus it seems that individual ridge segments bounded by transforms, could sample isolated mantle domains.

The recognition of mantle heterogeneity over a limited region of the MAR characterized by 'normal MORB' places constraints on the scale of the heterogeneities and the extent of melt

segregation in a convecting mantle. Allègre *et al.*¹⁶ recognized that the oceanic mantle is heterogeneous on the mineralogical scale and Zindler *et al.*¹⁸ identified long-lived mantle heterogeneity on a kilometre scale. Our data reveal that the mantle source for MAR is heterogeneous on a scale of tens of kilometres. Magma batches of a similar major and trace element geochemistry may thus be characterized by significantly different isotopic ratios. However, our data and other published data^{1–4} suggest that this mantle source is heterogeneous on the scale of tens to hundreds of kilometres, and that portions of the MAR are sampling long-lived heterogeneities that predate or coincide with the opening of the Atlantic Ocean. Evidence from the Kane Fracture Zone and other Atlantic Ocean fracture zones indicate that these domains may be segmented by major transforms.

The existence of isotopic heterogeneities in oceanic basalt glasses from adjacent sites implies that large scale magma chambers and regions of melt segregation do not exist in the upper mantle below the Kane Fracture Zone. We envisage that melting, in a convecting upper mantle, results in the separation of liquid from discrete pods and hence basalts from adjacent sites may display totally different pre-eruptive histories.

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Fluid evolution and graphite genesis in the deep continental crust

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Metamorphism in deep continental crust is often accompanied by carbon-rich, H_2O -poor fluids¹⁻⁵, and is characterized by the development of granulite facies mineral assemblages³⁻⁵. Graphite, a minor phase in many high-grade metamorphic rocks⁶⁻¹⁷, has also been recognized in recent xenoliths of deep crustal material¹⁸, demonstrating its presence in modern deep continental crust. Although graphite is most common in biotite schists, it has also been recorded in pyroxene gneisses, two feldspar-quartz pegmatites, marbles and quartzo-feldspathic gneisses^{11,15-19}. The presence of supracrustal sequences in granulite facies regions⁶⁻¹⁹ suggests that the latter developed through progressive metamorphism and dehydration of shallow level crustal material. Much of this material must have experienced a metamorphic recrystallization in the amphibolite facies, accompanied by the development of hornblende. Although graphite in the high-grade rock may develop from oxidation of organic material, this may not be the most common means for graphite genesis. In much of West Greenland¹⁴ and in southern Norway¹² the low-grade, hornblende-bearing material does not contain significant quantities of organic compounds, although graphite is found in higher-grade metamorphic equivalents. This strongly suggests that introducing CO_2 into deep crustal material leads to fluid evolution and graphite formation. I outline here the evolution of fluid composition in the C-O-H system which is in equilibrium with common crustal mineral assemblages. Thermodynamic data for CO_2 - H_2O mixtures²⁰ and mineral components²¹ are used to model the chemical changes in fluid composition which accompany introduction of CO_2 into various types of rock. The results demonstrate that graphite genesis is a direct consequence of deep crustal metamorphism in the presence of a CO_2 -rich fluid phase. In addition, the calculations demonstrate that the deep continental crust will not be a reservoir for large volumes of methane-rich gas.

Although the behaviour of fluids in deep continental crust is unknown, fluid inclusion studies imply that carbon-rich fluids may migrate through the crust over large distances (tens of kilometres)⁴. Here it is assumed that the fluid migration is accompanied by mixing of introduced CO_2 with *in situ* H_2O . Continuous addition (flow) of CO_2 ultimately removes H_2O from the deep crust. Thus, H_2O and CO_2 only mix along a migrating front of CO_2 -rich fluid. Below I examine fluid and mineral behaviour as this front migrates through biotite-magnetite-potassium feldspar (BMK) rocks.

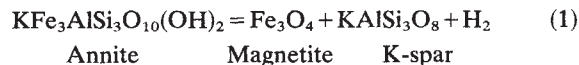
Rocks at deep crustal levels experience temperatures $>600^\circ C$ and pressures >7 kbar. In such conditions rocks are unlikely to possess any significant intrinsic pore space. Consequently, introduced or *in situ* fluids create pore space sufficient for their volume. Thus, fluid pressures are considered equal to lithostatic pressures, and no volume constraints are placed on the fluid.

Thermodynamic properties of CO_2 - H_2O mixtures were calculated from a modified Redlich-Kwong equation of state (MRK)²⁰. For the pure gases the derived fugacity coefficients are similar to those derived by Flowers²², but differ substantially from those presented by Ryzhenko and Volkov²³. H_2 , O_2 , CO and CH_4 fugacity coefficients were obtained from ref. 23.

Calculations were carried out to determine in what fluid compositions the BMK assemblage would be stable. These compositions were then compared with those for which graphite

is stable. This comparison determines whether graphite formation could result as a C-O-H fluid evolves in the presence of BMK.

The BMK assemblage was considered because it is common in granulite facies rocks. This assemblage defines hydrogen fugacity (f_{H_2}) through the equilibrium:



The equilibrium constant for equation (1) (K_1), derived from the thermodynamic data and SUPCRT computer program of Helgeson *et al.*²¹, yields

$$\log f_{H_2} = \log \left(\frac{a_{ann}^{bi}}{a_{mag}^{sp} \cdot a_{K-spar}^{feld}} \right) - 21.68 + 8.176 \log (T^\circ C) \quad (2)$$

where a_i^j is the activity of i in phase j (bi, biotite; ann, annite; sp, spinel; mag, magnetite; K-spar, potassium feldspar; feld, alkali feldspar).

Equation (2) was derived by fitting a logarithmic function to $\log k_1$ values which had been calculated at specified pressures and temperatures along chosen geothermal gradients. The small volume change of equation (1) at pressures >5 kbar and temperatures $>500^\circ C$ ($\Delta V_r < 0.88 \text{ cm}^3$) means that no pressure terms are needed in equation (2).

Solution of

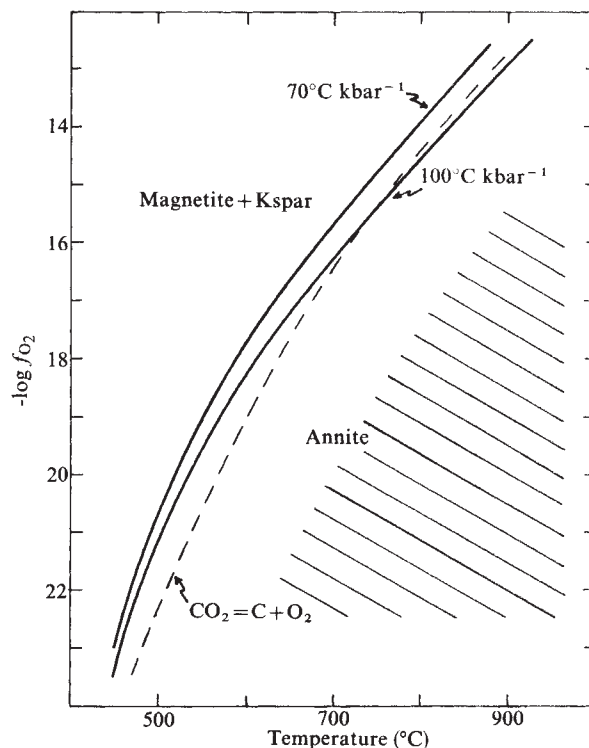


Fig. 1 $\log f_{O_2}$ plotted against T for the BMK assemblage at geothermal gradients of $70^\circ C \text{ kbar}^{-1}$ and $100^\circ C \text{ kbar}^{-1}$. Note the small pressure effect implicit in the curve location. The solid curves represent the case where $X_{H_2O} = 1.0$ and $X_{ann}^{bi} = 1.0$. The graphite-oxygen-carbon dioxide buffer curve is shown for reference ($100^\circ C \text{ kbar}^{-1}$, $P_f = P_{CO_2}$). The ruled region shows the CH_4 -dominated portion of the plane where $P_f = P_{CO_2} + P_{H_2O} + P_{CO} + P_{H_2} + P_{O_2} + P_{CH_4}$.

allows calculation of f_{O_2} in a BMK assemblage at a specified P , T (degrees kelvin) and X_{H_2O} . Solving $\log k_3$ for $\log f_{O_2}$, and substituting equation (2) for $\log f_{H_2}$ results in

$$\log f_{O_2} = 2 \left[\log (X_{H_2O} \cdot \gamma_{H_2O} \cdot P_f) + 21.20 - 8.176 \log (T - 273) - \left(\log \frac{a_{ann}^{bi}}{a_{mag}^{sp} \cdot a_{K-spar}^{feld}} \right) - \frac{12,510}{T \text{ K}} + 0.979 \log (T) \right] \quad (4)$$

X_{H_2O} is the mole fraction of H_2O in the fluid phase, γ_{H_2O} is the H_2O fugacity coefficient and P_f is the fluid pressure. Equation (4) allows f_{O_2} to be calculated in the presence of a fluid of variable composition which is dominated by H_2O and CO_2 .



allows calculation of f_{O_2} in the presence of graphite at a specified X_{CO_2} . Comparison of f_{O_2} values derived from equations (4) and (5), as a function of X_{H_2O} , provides a basis for outlining fluid evolution, graphite genesis and phase composition changes in the BMK assemblage.

All calculations were carried out along geothermal gradients. For comparison the results for the $100^\circ\text{C kbar}^{-1}$ and $70^\circ\text{C kbar}^{-1}$ geotherms are presented.

In the $\log f_{O_2} - T^*$ plane, equation (1) exhibits a positive slope similar to that of other oxygen buffers (Fig. 1). (Note that T^*

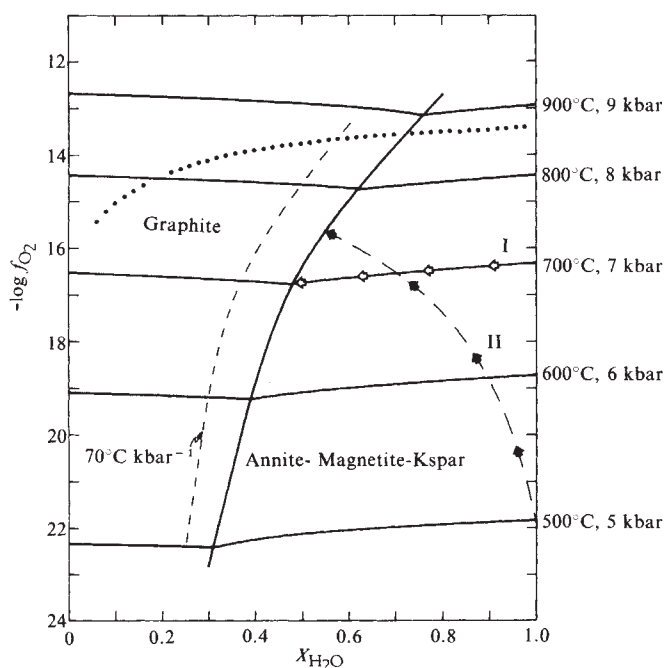


Fig. 2 Projection of BMK-fluid and graphite-fluid surfaces onto the $\log f_{O_2}/X_{H_2O}$ plane, from $\log f_{O_2}-X_{H_2O}-T^*$ space. The solid lines labelled in P and T are contours on the two surfaces ($100^\circ\text{C kbar}^{-1}$), the intersection of the surfaces being represented by the solid line which cuts the contours at a high angle. The intersection at a geothermal gradient of $70^\circ\text{C kbar}^{-1}$ is shown by the labelled dashed line. These contours and intersection are drawn for the case where $X_{ann}^{bi} = 1.0$ and assumes $X_{H_2O} + X_{CO_2} \cong 1.0$. The effect of lowered iron concentration in the mica is shown by the dotted line, which represents the contour for 700°C , 7 kbar , $X_{ann}^{bi} = 0.3$. Metamorphic pathways (I) (open arrows) and (II) (solid arrows) are discussed in the text.

represents specified pressure as well as temperature since all calculations are carried out along defined geothermal gradients.) However, the BMK assemblage is not an oxygen buffer because the solid and fluid phases are solutions; the location of the curve in the plane is specified only because the geothermal gradient and phase compositions are specified.

In $\log f_{O_2}-X_{H_2O}-T^*$ space, two surfaces can be drawn which represent the temperature-dependent, equilibrium fluid compositions for equations (1) and (5). Figure 2 shows these surfaces projected on to the $\log f_{O_2}-X_{H_2O}$ plane, and contoured for T^* . In this projection, the isothermic contours for the BMK surface trend to lower $\log f_{O_2}$ values as X_{H_2O} decreases. In contrast, the isothermic contours for the surface for equation (5) are at high $\log f_{O_2}$ values at low X_{H_2O} , and trend to low $\log f_{O_2}$ values at high X_{H_2O} . Because of the contrast in curvature, these fluid composition surfaces intersect to form a low $\log f_{O_2}$ trough in the $\log f_{O_2}-X_{H_2O}-T^*$ space, which is represented in Fig. 2 by the steeply sloping line. To the right of the intersection, the contoured surface represents the fluid composition in equilibrium with BMK assemblages lacking graphite; to the left of the intersection the surface represents the fluid composition in equilibrium with graphite-magnetite-potassium feldspar. The assemblage annite-magnetite-graphite-potassium feldspar can occur only along the intersection.

Additional components in the biotite will diminish annite activity and the BMK surface (Fig. 2) to higher $\log f_{O_2}$ values, and will displace the intersection to lower X_{H_2O} values. For an $X_{ann} = 0.3$, intersection of the surfaces occurs at X_{H_2O} of <0.1 , at 700°C , where X_{ann} was computed assuming $a_{ann}^{bi} = (X_{Fe}^{bi})^3$, after Wones²⁴. Addition of other components to magnetite and potassium feldspar shifts the BMK surface such that the intersection occurs at higher X_{H_2O} .

Two metamorphic pathways are possible in BMK-bearing deep crustal rocks into which CO_2 is being introduced. In the treatment below it is assumed that a geothermal gradient of $100^\circ\text{C kbar}^{-1}$ is present.

In case (I), CO_2 introduction takes place in isothermal and isobaric conditions. If the mica is pure annite, the $\log f_{O_2}-X_{H_2O}$ pathway will be that indicated by the open arrows in Fig. 2. As CO_2 is added to the system the modal abundance of mica will decrease, as will the oxygen fugacity, until the intersection trough is attained. At this point, graphite will precipitate from the fluid and annite will continue to react out until it has been eliminated from the assemblage. Once annite is removed, migration of the fluid composition along the isotherm will resume, increasing $\log f_{O_2}$ and X_{CO_2} until all H_2O is removed from the fluid.

Case (I) will be complicated if the mica is a mixed annite-phlogopite phase: then the mica composition and its modal abundance become critical equilibrium parameters. As CO_2 is added to the fluid the annite component in the mica reacts through equation (1), thus decreasing the activity of annite in the mica. As a result, the $\log f_{O_2}-X_{H_2O}$ pathway may differ substantially from the pure annite case as the BMK isotherms migrate to higher $\log f_{O_2}$ values as the activity of annite in the mica drops (Fig. 2). If the number of moles of annite per unit volume is small relative to the number of moles of CO_2 introduced at a given X_{CO_2} , the intersection of the two surfaces will rapidly migrate to very low X_{H_2O} values. In such conditions, graphite will probably not precipitate as the annite component will be removed from the mica before the fluid composition reaches the intersection trough. Such a system will have a phlogopite-rich mica coexisting with a CO_2 -rich fluid phase. If the number of moles of annite per unit volume is large relative to the number of introduced moles of CO_2 (a situation likely to occur when the pore volume is low), fluid evolution will approach that for the pure Fe-system where fluid migration can be traced along isotherms fixed in the $\log f_{O_2}-X_{H_2O}$ plane. Such a system will necessarily produce graphite coexisting with an Fe-Mg biotite.

Metamorphic pathway (II) considers the case in which CO_2 is introduced during increasing P and T . This case, for the pure Fe-system, is depicted by the dashed, solid-arrowed pathway in

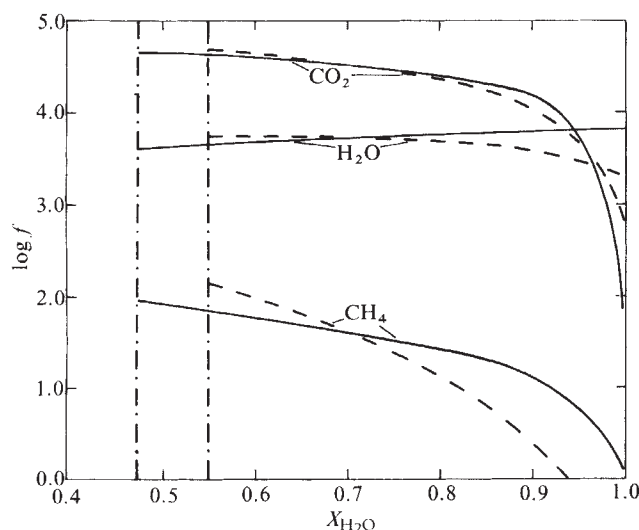


Fig. 3 Fluid evolution along metamorphic pathways (I) (isothermal, solid line) and (II) (polythermal, dashed line). Only H_2O , CO_2 and CH_4 are shown. O_2 can be deduced from Fig. 2; CO is slightly more abundant than CH_4 , H_2 slightly less abundant than CH_4 along both pathways. The vertical dash-dot lines mark the point of graphite precipitation from the fluid phase. Both pathways are calculated along the $100^\circ\text{C kbar}^{-1}$ geotherm, and are represented in Fig. 2.

Fig. 2. For a mixed Fe–Mg biotite the pathway would be much steeper than that depicted, as annite would be progressively removed from mica and the BMK surface would migrate to higher $\log f_{\text{O}_2}$ values. Whether or not graphite is produced in the Fe–Mg system would again depend on the relative volumes of annite and introduced CO_2 .

A comparison of the fluid species fugacities for these two metamorphic pathways (Fig. 3) demonstrates that in both cases a very rapid rise in f_{CO_2} occurs over an $X_{\text{H}_2\text{O}}$ range of 1.0–0.90. Methane, in both cases, also shows a significant rise, but it never becomes a dominant fluid species. However, the behaviour of H_2O and O_2 are dramatically different for the two cases. In case (I) both these fluid species exhibit a small but significant decrease in fugacity values before graphite precipitation. In case (II), however, both fluid species significantly increase their fugacity values as CO_2 introduction proceeds. In case (I), the H_2O behaviour will lead to progressive dessiccation of the enclosing rock as dehydration reactions occur in response to the lowering of $f_{\text{H}_2\text{O}}$. In case (II), increasing f_{O_2} may result in noticeable oxidation of the mineral assemblage. Solid-phase compositions will change in response to this increase in f_{O_2} through continuous reactions. The exact nature of the phase composition changes will be a function of the appropriate exchange equilibria at P and T , and can be evaluated from the $\log K$ expressions for the equilibria.

The BMK-graphite assemblage is not a fluid buffer because biotite composition changes in response to changes in fluid composition. Nevertheless, the assemblage forces fluid composition to lie along the $\text{C-CO}_2\text{-O}_2$ surface, placing severe constraints on fluid abundances, at P and T . If mineral compositions are known, fluid species abundances can be precisely determined. In typical deep crustal conditions (700°C , 7 kbar), the maximum possible $X_{\text{H}_2\text{O}}$ is 0.46 for the pure Fe system, although typical mineral compositions (for example, $X_{\text{ann}}^{\text{bi}} < 0.3$, $a_{\text{Ksp}}^{\text{feld}} > 0.7$, $a_{\text{mag}}^{\text{sp}} > 0.7$) allow a maximum $X_{\text{H}_2\text{O}}$ of 0.15. $\log f_{\text{O}_2}$ values are tightly constrained as they must fall on the $\text{C-CO}_2\text{-O}_2$ surface which exhibits little variation in $\log f_{\text{O}_2}$ at a specified P and T . If either biotite or graphite is missing from an assemblage, fluid composition cannot be quantitatively determined. However, the possible f_{O_2} and $X_{\text{H}_2\text{O}}$ values can be restricted to

specific ranges, at P and T and specified mineral compositions, because these values must lie along the appropriate isotherm on either the $\text{C-CO}_2\text{-O}_2$ surface (biotite absent) or on the BMK surface (graphite absent).

None of the explanations proposed for the genesis of abiotic graphite for a wide range of occurrences^{8,25–31} provides a means to develop graphite as a direct consequence of deep crustal metamorphism. The process outlined here, however, demonstrates that graphite will commonly develop in deep crustal settings as a result of CO_2 addition to mineral assemblages in which one of the O–H system fluid species fugacities is mineralogically controlled.

Biotic and abiotic graphite can be distinguished unambiguously by field criteria. In a terrain which has experienced a regional metamorphic event the presence of graphite in high-grade rocks, and the absence of carbon-bearing phases in lower-grade equivalents, for example, in southern Norway¹², is unequivocal evidence for an abiotic origin for the graphite.

The fluid evolution to be expected in such a setting (Fig. 3) is characterized by a very rapid rise in f_{CO_2} . Methane exhibits an increase in abundance, but never becomes a dominant fluid species, in the conditions examined here. Such considerations disprove suggestions^{32,33} that intermediate or deep-level continental crust may act as large reservoirs for methane-rich gases.

Although the fluid evolution outlined here relied on the features of only the BMK assemblage, similar observations can be expected from any mineral assemblage which specifies fugacity of a fluid species in the O–H system. Thus, most assemblages containing hydroxyl-bearing minerals and oxides will lead to graphite genesis, provided that the stability limits for these minerals are not exceeded at the $X_{\text{H}_2\text{O}}$ values compatible with graphite. The particular phase compositions which will be stable with graphite will depend on the geothermal gradient and the modal abundance of the solid phases, as well as on the total fluid volume affecting the rock.

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New hominoid skull material from the Miocene of Pakistan

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Neogene hominoid cranial material is regrettably scarce, especially from the middle and late Miocene. Between the 18-Myr-old virtually complete early Miocene *Proconsul africanus* skull from Rusinga¹ and the 3-4-Myr-old Hadar hominoid cranial material², the only significant large (non-hylobatid) hominoid facial or cranial specimens are those from the late Miocene Salonika in Greece³, Yassiören in Turkey⁴, Lufeng in China⁵, the possibly middle Miocene site of Moroto in Uganda⁶ and the new facial-mandibular piece from late Miocene deposits in Pakistan reported here. The specimen is a *Sivapithecus indicus*⁷ adult, probably male, and consists of most of the left side of the face including a small portion of the frontal bone, the zygomatic arch and temporo-mandibular joint, the maxilla, a virtually entire mandible and the complete dentition. The fossil, catalogued as GSP 15000, is the property of the Government of Pakistan and is at present on loan for study at Harvard University.

The face was found during the 1979-80 field season of the Geological Survey of Pakistan-Harvard (then Yale) Peabody Museum Siwalik (GSP-HPM) research project on the Neogene of the Potwar Plateau of Pakistan (co-directed by Dr S. M. Ibrahim Shah and myself). The fossil was discovered by Mark Solomon and Dr John Barry and prepared at Yale by Horace French and Rizwana Raza. It comes from GSP-HPM locality 410 near the village of Kaulial in the Potwar Plateau⁸. Locality 410 is in the Dhok Pathan Formation⁹, the locality itself being composed predominantly of red silts, representing overbank deposits¹⁰. The partial skull was probably not transported far after the animal's death, and was probably scavenged after death. The only other directly associated fossils are a few small mammals—murids, a flying squirrel and a dormouse. However, we have an abundant mammal fauna from many localities at the same stratigraphic level as the face, the so-called U sandstone level, after a prominent marker horizon⁸.

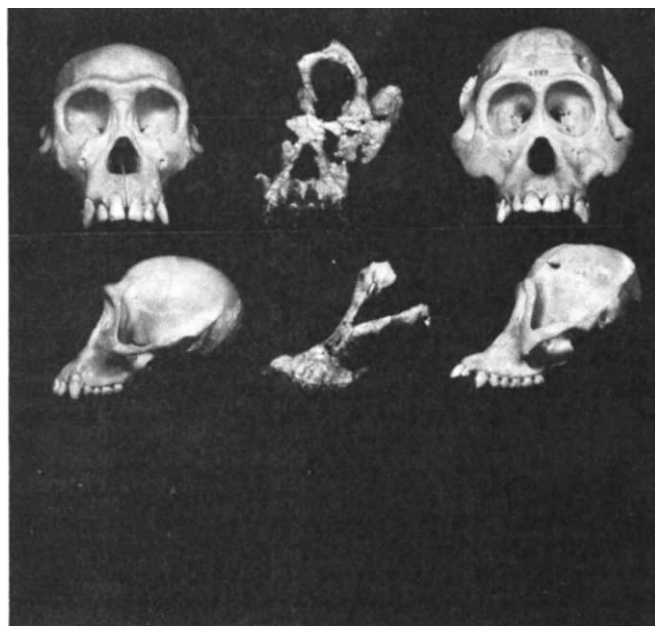


Fig. 1 Crania of *Pan* (left), GSP 15000 and *Pongo* (right).

Table 1 Facial, maxillary and mandibular measurements

Nasal height		84.0
Bizygomatic breadth	est.	75.0
Alveolare to nasospinale		26.0
Nasal aperture height	est.	29.0
Nasal aperture breadth		21.0
Nasal bone length	est.	58.0
Orbital height	est.	43.0
Orbital breadth		34.5
Zygomatic depth	est.	43.0
Orbito-alveolar height	est.	63.0
Minimum biorbital breadth	est.	9.5
Biorbital breadth across lacrymal crests	est.	10.5
Palatal length		83.0
Palatal breadth at C		31.0
Palatal breadth at M ²		32.5
Incisive foramen breadth	est.	2
Palatal depth at C	est.	8
Palatal depth at M ²	est.	17
Alveolar breadth at C		59.0
Alveolar breadth at M ²		59.0
Symphysis	Depth	est. 42
	Thickness	est. 20
Corpus at P ₄	Depth	37.0
	Thickness	16.0
Corpus at M ₃	Depth	34.0
	Thickness	28.5
Ramus	Notch height	est. 88.0
	Condylar height	est. 108.0
	Length	est. 60.0

For definitions of parameters measured, see refs 4, 6, 17.

The U level is ~8 Myr old, based mainly on magnetostratigraphic correlations^{11,12}. A brief period representing perhaps less than 0.1 Myr has been sampled intensively at that level¹³. No significant plant remains are known, but we have tentatively reconstructed palaeogeography, habitats and faunal communities by a variety of geological and faunal techniques^{8,10,13}. The late Miocene sediments were laid down by two major aggrading river systems, with many associated smaller streams, in an area of predominantly low relief. The habitat was probably a complex mosaic of forest, woodland and grassland, probably with a rich shrub layer. This would explain the abundance of browsing mammals, along with grazers, in the community^{8,14}.

Sivapithecus indicus is well represented at the U level by jaws and teeth, and a few unassociated postcranial elements probably also represent the same species⁷. If these assignments are correct the species was approximately chimpanzee-sized and more hominoid-like than cercopithecoid-like in overall anatomy; it was probably predominantly arboreal.

The new specimen is unusually complete for this range of time, comprising an upper face, palate, and mandible with a complete adult dentition (Figs 1, 2)¹⁵. The maxilla above the palate is preserved bilaterally and completely outlines the nasal aperture. Most of the remainder of the right side of the face is missing, except for the base of the malar process and a wedge of nasal process extending almost up to the frontal bone. In the midline and on the left side of the face, the specimen includes the nasal bones, almost complete orbital margin including the lower portion of the frontal bone, and the lateral edge of the face marking the anterior border of the temporalis muscle. The zygomatic bone is well preserved on the left and articulates with a segment of the zygomatic process of the temporal bone. A large fragment is missing from the midface on the left in the area of the zygomatico-maxillary suture. A separate fragment of the left temporal bone includes a complete articular fossa with a prominent postglenoid process. The lateral extent of the articular fossa is complete while medially the piece ends just beyond the articular surface. The mandibular corpus is bilaterally preserved back to the third molars, with a buccinator line well preserved on the right but mostly missing on the left. The specimen is broken and somewhat crushed on the left of the midline. Most of the left ramus is present, including a fragment of condyle, and the right ramus is also relatively complete.

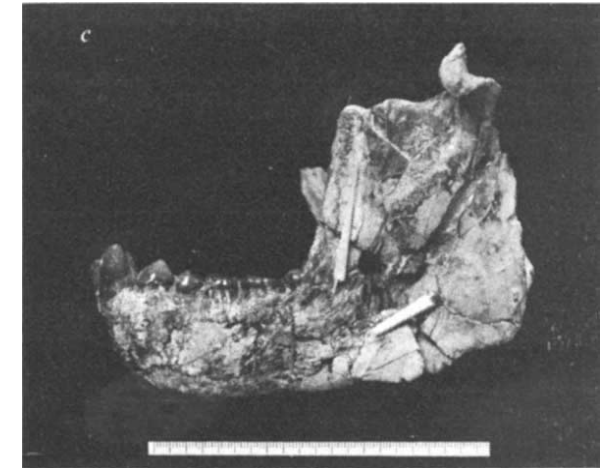
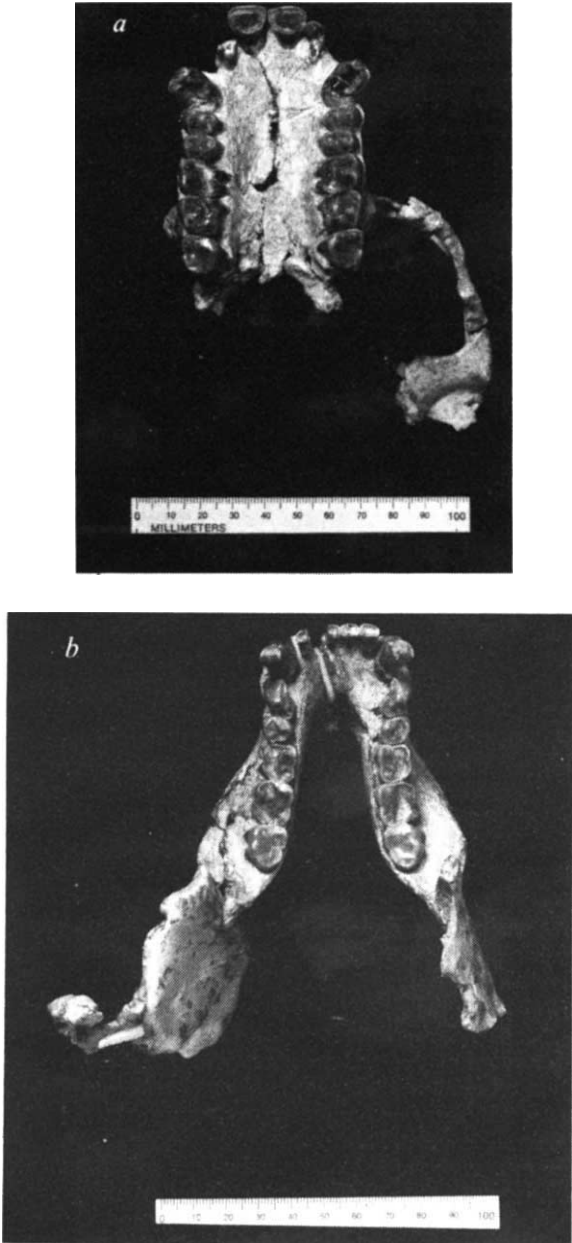


Fig. 2 a, Palatal view of GSP 15000; b, occlusal mandibular view of GSP 1500; c, lateral mandibular view of GSP 15000.

The overall impression is of a delicate face combined with robust jaws and teeth (see Table 1 for measurements). The face is deep from frontal to tooth row and concave in profile. The supraorbital tori are small and the frontal profile steep; there is no frontal sinus. The orbits are ovoid, with their longest dimension vertical, and are separated by a narrow interorbital region. The midfacial length from orbit to nose is greater than that in *Pan* or *Pongo*. The premaxillary region is prow-like and curved, and the incisors quite strongly procumbent. The zygomatic is gracile, and the temporo-mandibular joint shallow and crescentic, with the convexity facing anteriorly; it is ape-like, and similar to that of *Pongo*. The mandible is robust and deep, with well developed posterior symphyseal buttressing; the rami are high and vertical to the corpus.

Table 2 gives dental measurements of GSP 15000. Judging from canine size this is a male. Overall wear on the dentition is very heavy and most occlusal detail is therefore lost; in some cases dentine is extensively exposed. Unworn *Sivapithecus indicus* cheek teeth are high crowned and low cusped, with few occlusal wrinkles, and have thicker enamel than almost all Neogene hominoids with the exception of *Australopithecus*⁷. Wear on the incisors and canines of GSP 15000 is heavy; canines are blunted, and the small upper lateral incisors oddly worn, presumably by occlusion with the lower canines. This suggests extensive anterior tooth use in non-masticatory activity. The maxillary sinus floors descend mostly to the root tips, although posteriorly sinus pockets extend between the roots.

Sufficient frontal bone is preserved and enough known of the position of temporo-mandibular joint and adjacent temporal bone to reconstruct tentatively the anterior half of the brain

Table 2 Dental measurements

		Left		Right		Left		Right	
I ¹	MD		12.3		12.4	I ₂	LaLi		7.2
	LaLi		9.0		9.1		MD		6.5
I ²	MD	est.	6.0		—		Lali	8.7	8.8
	LaLi		—		—	C̄	Max	13.7	13.8
C	Max		16.0		15.5		Tr	9.8	9.3
	Tr		12.3		11.6	P ₃	Max	13.9	14.1
P ³	MD		8.6		8.7		Tr	7.9	7.9
	BuLi		11.8		12.1	P ₄	MD	8.8	8.9
P ⁴	MD		8.0		8.4		BuLi	10.4	10.0
	BuLi		12.0		12.3	M ₁	MD	12.4	12.6
M ¹	MD		12.5		12.4		BuLi	11.3	11.4
	BuLi		13.0		—	M ₂	MD	14.3	14.7
M ²	MD		12.8		12.8		BuLi	12.9	13.4
	BuLi		14.4		14.0	M ₃	MD	14.3	14.8
M ³	MD		12.4		12.6		BuLi	12.7	12.4
	BuLi		13.4		14.0		Talonid	11.5	11.2
I ₁	MD		—		5.7				

For definitions, see ref. 6. Note that all lengths are estimates, and greatly affected by wear.

case. This was hafted high on the face, as in *Pongo* but unlike *Pan*; brain volume was probably in the ape range. In this feature, and in facial profile, jaw joint morphology, malar morphology, orbital shape and disposition, and overall palatal shape, the specimen is quite orang-like. In other features the two species differ; for example, in tooth morphology and wear, and in the considerable fossil midfacial length and ramus height.

Compared with the few other Miocene facial or cranial specimens known, GSP 15000 differs from *Proconsul africanus*¹ and *Proconsul nyanzae*¹⁶ in being more like living pongids, and also differs from the possibly middle Miocene palate and face from Moroto in Uganda⁶. Among other late Miocene hominoids, it is most like the *Sivapithecus* face from Yassören⁴ and less like *Ouranopithecus* from Ravin de la Pluie³. However, detailed comparisons with all these specimens, and with those from Lufeng⁵ in China and from the Pliocene deposits at Hadar, Ethiopia², have not yet been completed.

As reconstruction and analysis of GSP 15000 continues, attention is being paid to the biomechanical meaning and potential functional significance of facial, mandibular and dental features; this involves detailed understanding of equivalent parts in modern systems. As these studies proceed it should be possible to develop a clearer understanding of the significance of the attributes frequently used in taxonomic and phylogenetic

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analyses. In particular, this will help evaluate the 'primitive' or 'derived' nature of character states.

Meanwhile, there are several similarities between GSP 15000 and *Pongo* that may turn out to be shared derived features. I doubt that *S. indicus* itself is ancestral to *Pongo*; however, I consider it likely that a similar late Miocene species, probably an Asian one, was. To what extent *S. indicus* resembles the last common ancestor of all living pongids and hominids is unclear. There are a few resemblances in mandibular and dental morphology (thick enamel, megadonty, mandibular robusticity) between Pliocene *Australopithecus* and *Sivapithecus* and similar late Miocene species, which may be primitive retentions. If so, in those features at least, the African pongids would be derived rather than primitive, which would have important consequences for the interpretation of hominid origins. However, it would be better to wait until more complete late Miocene hominoid material is recovered, especially from Africa, before constructing evolutionary stories.

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Body temperature changes during the practice of g Tum-mo yoga

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Since meditative practices are associated with changes that are consistent with decreased activity of the sympathetic nervous system¹⁻⁷, it is conceivable that measurable body temperature changes accompany advanced meditative states. With the help of H.H. the Dalai Lama, we have investigated such a possibility on three practitioners of the advanced Tibetan Buddhist meditative practice known as g Tum-mo (heat) yoga living in Upper Dharamsala, India. We report here that in a study performed there in February 1981, we found that these subjects exhibited the capacity to increase the temperature of their fingers and toes by as much as 8.3 °C.

g Tum-mo yoga is a form of meditation which allegedly allows its practitioners to alter body temperature. Previously, only subjective descriptions of this phenomenon existed: "The neophytes sit on the ground, cross-legged and naked. Sheets are dipped in icy water, each man wraps himself in one of them and

must dry it on his body. As soon as the sheet has become dry, it is again dipped in the water and placed on the novice's body to be dried as before. The operation goes on in that way until day-break. Then he who has dried the largest number of sheets is declared the winner of the competition"⁸.

During the practice of g Tum-mo yoga, 'prāna' (literally, 'wind' or 'air') is withdrawn from the scattered condition of normal consciousness and is made to enter into the 'central channel' inside the body. Then, through the alleged dissolution of these winds in the central channel, the 'internal heat' is ignited. The physiological changes are, therefore, a by-product of a religious practice.

Each of the three monks in the present investigation had spent more than 6 years practising g Tum-mo daily and had lived most of the past 10 years in near-isolation in unheated, uninsulated stone huts ~4 × 7 m. The floors were earthen and the roofs made of large slate slabs or flattened tin cans. The huts were in isolated locales in the foothills of the Himalayan Mountains at altitudes of 1,800–2,800 m, outside the town of Upper Dharamsala. The first two subjects were studied in their hermitages while the third was studied in a hotel room in Upper Dharamsala. Each participated in the investigation after being asked to do so by H. H. the Dalai Lama. Verbal informed consent was obtained from the three subjects through our translator (J.H.).

Skin temperature was measured in the area of the navel and lumbar region, the chest (nipple), left forearm, left fifth finger nailbed, left calf, left fifth toe nailbed and forehead using small disk thermistors (Yellow Springs Instrument Corporation (YSI), model 427) that were attached to the skin with plastic tape. Rectal temperature was monitored by means of a YSI rectal catheter probe inserted 10 cm beyond the anal sphincter. Air temperature was measured by YSI thermistor air probe (model 405) as well as with a conventional sling psychrometer which was used to measure psychrometric wet bulb temperature to calculate relative humidity. All wires were connected to a multi-channel (YSI) switch box (model 4002) and connected to a YSI wide-range Telethermometer (model 42) with a 60 °C range. Heart rate was monitored with a San-EI (model 2D16) Pulse-

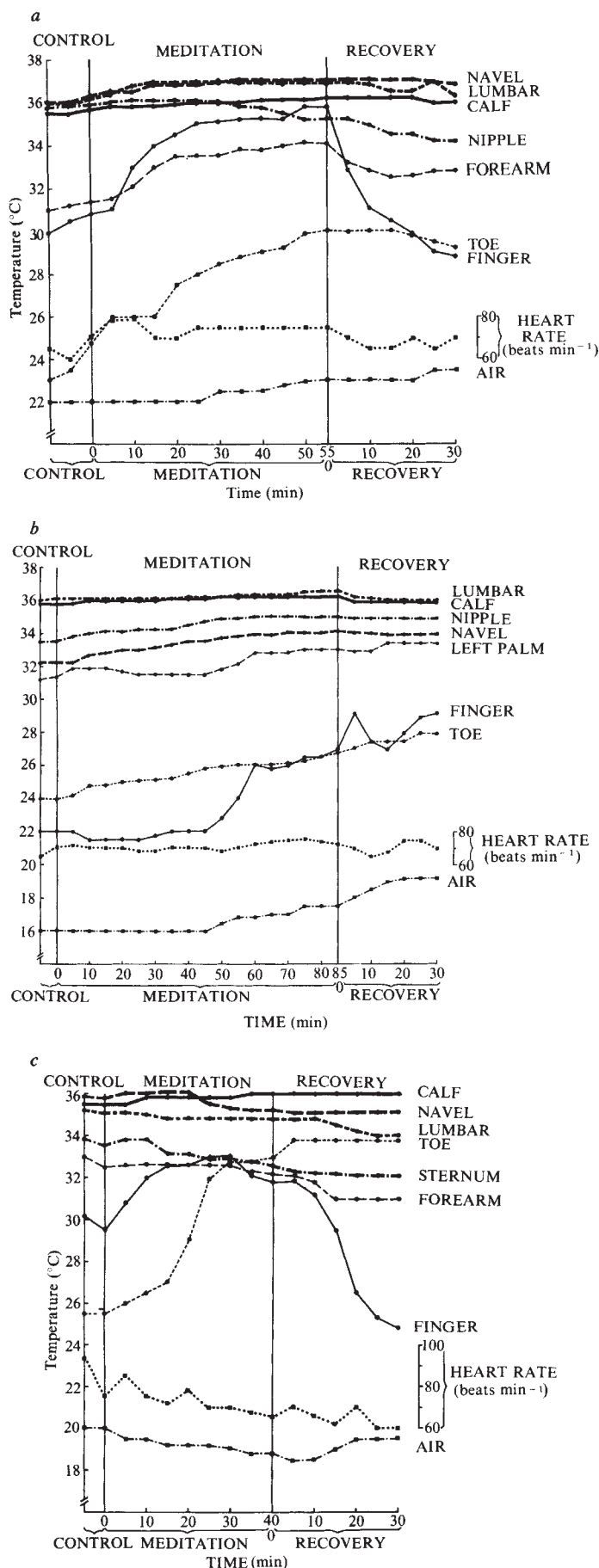


Fig. 1 Skin and air temperature and heart rate changes before, during and after the practice of g Tum-mo meditation in subjects G.J. (a), J.T. (b) and L.T. (c).

meter monitor which used a digital probe to sense each pulse of blood flowing into the thumb. Measurements were taken sequentially and tabulated every 5 min during control, meditation and recovery periods.

The first subject, Ven. G. J., aged 59 yr, sat in the cross-legged 'Lotus' posture. During a 55-min period of g Tum-mo meditation, his finger temperature increased 5.9°C and his toe temperature 7.0°C (Fig. 1a). In the recovery period, finger temperature returned to baseline levels, while toe temperature, with the toes protected against heat loss by the lotus position, remained elevated. Navel and lumbar temperatures increased by 1°C during the meditative period. Rectal temperature was unchanged. Air temperature slowly increased from 22.0 to 23.5°C during the hour. Heart rate increased slightly, by 2 beats per min, during the meditative period and then returned to baseline levels.

Finger temperature in the second subject, Ven. J. T., aged 46 yr, increased 7.2°C, the highest levels being recorded in the recovery period at a time when the subject claimed that, although he had stopped meditating, the subjective experience of g Tum-mo heat had continued (Fig. 1b). A similar pattern with a 4.0°C increase was noted in the toe temperature. Navel temperature increased 1.9°C and nipple temperature 1.5°C. Rectal temperature was unchanged. Air temperature slowly increased within the hut from 16°C to 19.2°C, the most marked changes occurring late in the recovery period. Heart rate was essentially unchanged throughout.

The third subject, Ven. L. T., was 50 yr old. Because his g Tum-mo changes started any time he was in a sitting posture, baseline measurements were taken in the upright posture. We first performed measurements on this subject outside his hut. However, high radiant heat temperatures hampered skin temperature measurements and since our visit coincided with the celebration of the Tibetan New Year, when the monk walked to Upper Dharamsala to listen to a lecture given by H. H. the Dalai Lama, we studied him again in a cool hotel room. His finger temperature rose 3.15°C while toe temperature increased 8.3°C (Fig. 1c). There were no other large changes in skin temperature. Rectal temperature was unchanged. Air temperature within the hotel room decreased during the meditation period from 20 to 18.5°C, and then rose to 19.5°C in the recovery period. Heart rate decreased after assuming the sitting position and continued to fall slowly throughout the experiment.

The subjects in the current experiment exhibited a greater capacity to warm fingers than has been previously recorded during hypnosis and after biofeedback training⁹⁻¹⁵. Although it is possible that the practitioners had learned to increase their metabolism to produce more body heat, this seems unlikely. Even though they were all lean, the monks claimed not to require more than a 'normal' amount of food. Furthermore, their resting heart rates were within normal limits. The most likely mechanism to account for the increase in finger and toe temperature is vasodilation. Although no direct measurements of peripheral blood flow were made, others have calculated that during the simple meditative process of transcendental meditation, there is a 44% increase in nonhepatic, nonrenal blood flow¹⁶ and they hypothesized an increase in skin or cerebral blood flow or both.

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Preferences for cousins in Japanese quail

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Early experience affects the mating preferences of many birds and mammals^{1,2}. The plasticity of behaviour is such that individuals reared with a member of another species may subsequently choose to mate with that species^{3–6}. Recent evidence suggests that the most strongly preferred mates are slightly different from individuals that are familiar from early life^{7–9}. The implication of these findings is that an individual is able to strike an optimal balance between inbreeding and outbreeding by learning about its immediate kin and mating with a member of the opposite sex that is slightly different from its immediate kin^{1,10}. What such a balance might amount to in practice has previously been uncertain. I report here that Japanese quail of both sexes, having been reared with their siblings, subsequently prefer a first cousin of the opposite sex.

The Japanese quail (22 males and 13 females) tested in this experiment were obtained from eggs laid by four different pairs in our laboratory colony. Eggs from a given pair were collected over 2-week periods and placed simultaneously in a separate compartment in an incubator. When transferred 13 days after the start of incubation to a hatching incubator, eggs were again

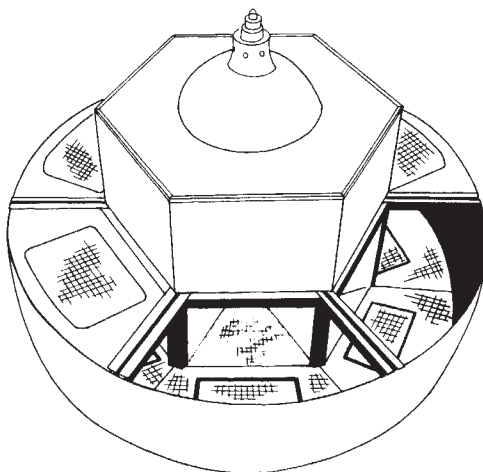


Fig. 1 Apparatus used for testing preferences of adult Japanese quail. Stimulus birds were put singly in the inner compartments, which were painted white and lit from above. The outward-facing windows were fitted with one-way screens. A test bird could move freely round the black unlit outer part of the apparatus. A pedal in front of each window operated a microswitch.

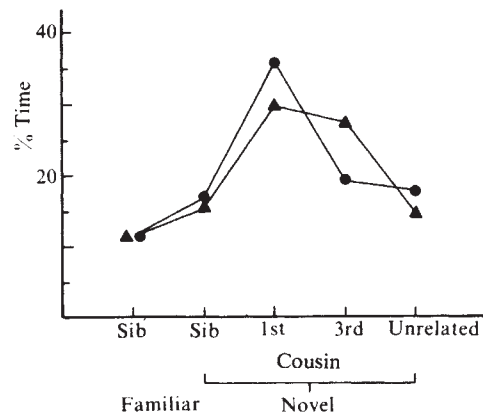


Fig. 2 The mean percentage time spent by adult Japanese quail near members of the opposite sex that were either familiar siblings (Sib), novel siblings, novel first cousins, novel third cousins, or novel unrelated individuals. The chance level is 20%. ▲, Males ($n = 22$); ●, females ($n = 13$).

kept in separate families and hatched separately. Within a day of hatching in the dark, the chicks were transferred to rearing pens and kept in true families in the same conditions as those used in previous experiments^{7,9}. The birds were isolated 30 days after hatching. Testing started at 60 days, when the quail were sexually mature.

The pedigrees of the quail in the colony were known for at least four generations and pairings had been carefully arranged. It was possible, therefore, to test all birds with a variety of members of the opposite sex that were of different degrees of relationship but of the same age. In addition, some true siblings were reared separately so that when the birds were tested, they were given choices between familiar and novel siblings, novel first and third cousins, and novel unrelated members of the opposite sex.

The apparatus used for testing the adult quail is shown in Fig. 1. The compartments in which the stimulus birds were placed faced outwards from the centre of the apparatus. The inside walls were painted white and the outside wall consisted of a one-way screen. The outward-facing windows were used so that the test bird was not visible to the stimulus birds. The outer runway was painted black and the test bird could move freely from one window to the next. To eliminate possible bias, when a test bird was initially introduced into the apparatus, it was placed in front of a compartment containing a different stimulus bird from the one tested previously. Furthermore, food and water for the test bird were placed on the outside wall opposite the compartment of every stimulus bird. Each test session lasted 30 min. Pedals which triggered microswitches were placed in front of the compartment of each stimulus bird. The time spent in front of each of these stimulus birds was recorded automatically and expressed as a percentage of the total time spent in front of all five types of stimulus bird.

Figure 2 shows the mean percentage duration spent in front of each category of stimulus bird by both adult males and females. Table 1 gives the scores for each individual. The males were slightly more ready than females to spend time near a third cousin of the opposite sex. However, in this sample at least, the behaviour of the two sexes were statistically indistinguishable and, for the purpose of the analysis, these data were pooled. First, all the data were examined using the Friedman test and the overall deviation from chance was found to be statistically significant ($\chi^2 = 10.07$, $P < 0.05$). Second, the time spent near each category of stimulus bird was compared with the time spent near each other category of stimulus bird using the Wilcoxon matched-pairs test. The time spent near novel first cousins was significantly greater than that spent near both familiar and novel siblings ($z = 3.02$, $P < 0.01$ and $z = 2.23$, $P < 0.05$, respectively). The time spent near novel first cousins was also

Table 1 Percentage time spent by adult Japanese quail near members of the opposite sex

Sex	Subject	Familiar sibling	Novel sibling	Novel first cousin	Novel third cousin	Novel unrelated
Male	1	2.3	29.4	41.7	22.5	4.0
	2	42.4	0.0	49.6	0.0	8.0
	3	0.0	4.0	11.8	3.4	80.8
	4	11.7	33.7	23.2	9.6	21.7
	5	14.5	19.1	32.8	33.6	0.0
	6	0.3	43.6	2.1	30.5	23.5
	7	3.4	0.1	26.7	50.8	19.1
	8	0.0	0.4	87.4	0.0	12.1
	9	0.0	0.0	0.1	97.2	2.7
	10	52.4	0.0	1.6	46.1	0.0
	11	3.9	88.3	7.6	0.0	0.2
	12	0.0	0.2	24.8	75.1	0.0
	13	1.0	3.3	30.9	63.8	1.0
	14	0.0	1.3	0.0	94.4	4.3
	15	59.2	0.0	0.0	35.2	5.7
	16	8.5	52.4	13.5	15.5	10.1
	17	17.1	4.5	78.4	0.0	0.0
	18	23.0	14.3	17.8	12.7	32.2
	19	0.0	72.5	26.3	1.2	0.0
	20	10.1	0.0	89.9	0.0	0.0
	21	0.0	5.9	52.2	8.9	33.0
	22	2.9	0.1	34.2	0.1	62.7
Female	23	20.6	6.0	0.3	67.8	5.4
	24	12.8	25.6	43.3	11.8	6.6
	25	0.0	3.4	96.6	0.0	0.0
	26	55.9	26.3	7.3	3.2	7.2
	27	9.6	5.5	13.6	49.1	22.2
	28	0.5	0.0	63.6	0.1	35.8
	29	0.0	23.0	42.6	10.5	23.9
	30	0.0	0.0	7.7	82.0	10.2
	31	30.9	13.4	20.0	8.2	27.4
	32	4.6	61.3	4.3	7.9	22.0
	33	0.0	17.2	77.2	2.8	2.8
	34	14.0	20.2	2.9	9.0	53.9
	35	0.0	0.0	86.1	0.0	13.9
No. showing strongest preference for category		4	6	12	9	4

significantly greater than the time spent near unrelated individuals ($z = 2.18$, $P < 0.05$).

Of course, remaining near a member of the opposite sex is not the same as attempting to mate with it. However, other experiments have shown that, in adult male Japanese quail, the time spent near a female in a choice test is strongly linked to the copulation preference⁷. Furthermore, the males in the present experiment were observed to court the females in the choice tests. Finally, I have found in other experiments that birds show no consistent preferences for members of the same sex.

Despite the clear overall preference for first cousins, the data were highly variable and only 12 of the 35 birds spent most time near a first cousin (see Table 1). The variability was expected as the degree of relationship between two individuals only indicates the probability that the two share heritable characters. I have confirmed this by direct measurement of the quails' plumage. Although, for example, first cousins are on average more like each other than unrelated individuals, unrelated birds may resemble each other more closely than some first cousins. Knowledge by a bird of a sibling's appearance must necessarily be an imperfect guide to what a first cousin will look like.

The most preferred category of relations will probably depend on the degree of outbreeding of the population⁹. The quail used here were relatively inbred and 'unrelated' birds were physically rather similar to each other. In populations that are physically more variable than our laboratory quail, the most preferred member of the opposite sex might be more closely related than a first cousin. In contrast, the effect of restricted experience in a laboratory experiment might have meant that the birds were more ready to escape from novelty than would be the case for quail growing up in a natural environment. Therefore, in a

free-living population, the effects on sexual preferences of greater variation in the plumage of the population may cancel out those of more varied experience.

In many human societies, marriage to a first cousin is common¹¹. This apparent similarity between the behaviour of humans and quail should be noted. Humans may strike a balance between the costs of inbreeding and those of outbreeding^{10,12} and in doing so, may rely on their early experience in a way similar to quail. Clearly, many factors deriving from culture and individual experience affect human mating preferences, but one influence may well be an attraction towards a member of the opposite sex who differs, but not to a large extent, from well known, closely-related members of the family.

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Early auditory experience modifies sound localization in barn owls

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The auditory system localizes sounds by comparing the timing and intensity of sounds arriving at the two ears, and associating specific binaural differences with directions of sounds in space. Thus, location for the auditory system, like stereoscopic depth for the visual system, is a percept created in the brain by a comparison of inputs from two receivers. We considered whether the neural mechanism underlying sound localization is determined entirely genetically, or if it is modified and regulated by auditory experience. The influence of experience on sound localization has been examined previously in various species, including man, with contradictory results¹⁻⁹. For our study we used the barn owl (*Tyto alba*) as an experimental model because it localizes sounds with extreme accuracy^{10,11}. By placing a plug in one ear, we disrupted the owl's binaural localization cues and induced large errors in sound localization. We report here that young owls adjusted to the altered cues and regained normal localization accuracy over a period of weeks. However, as the owls aged, their rate of adjustment slowed, and beyond 6-7 months of age, their capacity to adjust was lost or greatly reduced. This plasticity of the sound localization mechanism early in life thus enables the auditory system to establish precise correlations between binaural cues (which vary between individuals) and directions of sounds in space.

The localization cues used by the barn owl are those used by man—interaural differences in intensity and time¹²⁻¹⁴. However, because of a vertical asymmetry in the positions of the owl's ears, high frequency sounds are loudest in the right ear when they originate above and to the right, and loudest in the left ear when they come from below and to the left¹⁵. Behavioural and physiological studies demonstrate that owls use interaural intensity cues to localize the elevation of sounds, and both interaural time and intensity cues to localize the azimuth^{13,14}.

In our experiments, we occluded ears by suturing a foam rubber plug into the auditory canal. The effect of such a plug on the auditory signal was determined in one bird by measuring the sensitivity and phase of cochlear microphonic responses before and after the treatment. The plug attenuated the signal by 30-55 dB from 1 to 10 kHz depending on frequency, and altered the phase of each frequency. Thus, both interaural intensity and timing cues were severely disrupted by this procedure.

The experiments were done in a darkened anechoic chamber with the owl perched in the centre. Sound stimuli were generated by a 4-cm speaker that could be moved to any position around the owl by remote control. Travelling with the speaker and centred in its cone was a 3-mm light bulb that provided a light stimulus. The behavioural paradigm for measuring sound localization error took advantage of a natural head orienting response: on hearing a sound the owl turns its head in a rapid flick or saccade, bringing the source of the sound into a frontal region of maximum spatial acuity for the auditory system¹⁰. This movement also brings the visual axes of the eyes, which are immobile in their sockets, in line with the target. A normal owl orients its head almost identically to either a sound or a light stimulus. In these experiments, a difference in the orientation of the head to sound as opposed to light was taken as an error in sound localization (Fig. 1). The behavioural paradigm simply required the owl to orient its head in response to either

the sound or light stimulus, for which it received a food reward. The reward was not contingent on the accuracy of the response, only on its crispness and appropriate latency. To quantify the position of the head, a small mirror was attached to a clip cemented to the skull, and a collimated source of IR light was directed at the bird. The mirror reflected the IR beam onto a grid of 0.5° increments in a horizontal polar coordinate system. The location of the beam on the grid was monitored using a video camera and stored on video tape for subsequent analysis.

The sound target consisted of repetitive noise bursts of variable duration; the light target was a constant dim glow of the bulb. In both cases the stimulus was presented for up to 2 s. If the owl did not respond, the stimulus was turned off, followed by a waiting period of 30 s. Typically, however, the owl responded immediately with a saccadic head movement and sustained fixation. The stimuli were maintained long enough to allow the owl to maximize its accuracy. The coordinates of the head orientation were then recorded, a reward light activated, and a food reward delivered. After every trial the speaker (with light bulb) was moved to a new location. The owl received no feedback regarding the accuracy of its responses during these tests.

Five baby owls were used, four as experimental subjects and one as a control. All were kept in identical holding cages and allowed out daily for training and exercise. The auditory experience they received consisted of self-generated noises when active in their cages, and the sounds in the laboratory when allowed out for exercise. At 4 weeks of age we occluded the left ear of owl 1 and the right ears of owls 2 and 3; the right ear of owl 4 was occluded only at 27 weeks. Owl 5, which served as a control and was never plugged, demonstrated the accuracy and precision that can be expected from a normal owl under our experimental conditions. From four measurements made at 18 weeks of age, the mean error of owl 5 ranged in magnitude from 0.8° to 1.4° in azimuth and from 0.4° to 1.7° in elevation, with respective standard deviations of 1.4-2.3° and 1.3-2.7°. These values are almost identical to the localization accuracies of two adult owls that were studied intensively in previous behavioural experiments, using a different measuring technique¹⁰.

From these results, we defined normal localization accuracy as an error of $\leq 2^\circ$ in azimuth and elevation (indicated by broken

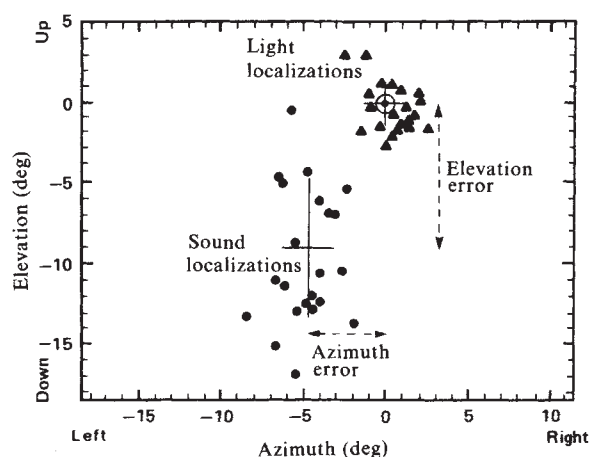
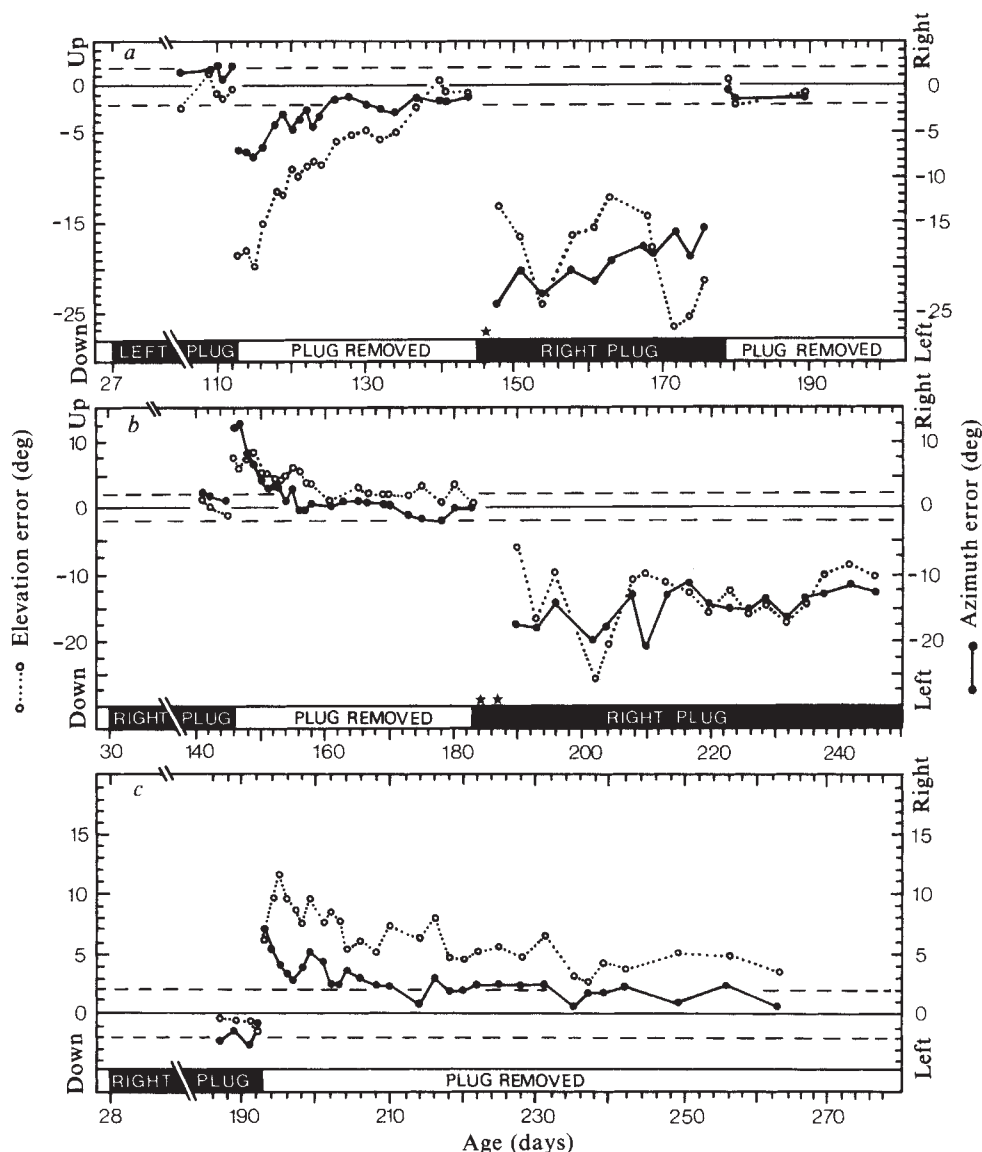


Fig. 1 The distribution of head orientations to sound and light stimuli recorded in a single test session. Each symbol represents a fixation point in response to a sound (●) or light (▲) stimulus. The data were normalized for the location of the stimulus by subtracting the coordinates of the speaker or light bulb from the coordinates of the elicited head orientation. This gave two sets of data points, one for sound and the other for light localizations. The mean and standard deviation of each distribution is indicated by the crossed lines. Sound localization error was defined as the difference in azimuth and elevation of these means. The scatter (standard deviation) of the responses served as a measure of the precision of localization. These data were taken from owl 1 at 120 days old, 1 week after its plug had been removed. In a fully adjusted owl, these point distributions would superimpose almost perfectly.

Fig. 2 Adjustment of sound localization accuracy after inserting and removing monaural plugs in three owls: *a-c* are owls 1–3, respectively. The localization error of each owl is plotted as a function of its age. The azimuth (●) and elevation (○) components of the error, derived as shown in Fig. 1, are plotted separately. The bar at the bottom of each graph shows the auditory history of the owl. The limits of what we considered to be normal accuracy are indicated by the broken lines. The asterisks on day 146 for owl 1 and on days 184 and 187 for owl 2 represent sessions in which the owls were unable to localize the sound target.



lines in Fig. 2). An owl was considered to have reached normal accuracy when its error fell within these limits on two consecutive days. (In presenting these data, R signifies a localization error to the right of the source, L to the left, + above and – below.) Owl 1 (left plug) was the youngest bird to be trained and tested. At 16 weeks old, having been plugged for 12 weeks, the median error ($\pm$ s.d.) of this owl was R $2.0^\circ \pm 3.1^\circ$ in azimuth and $-1.3^\circ \pm 3.5^\circ$ in elevation (Fig. 2a). Apparently, owl 1 had learned to localize with normal accuracy despite the severe monaural impairment caused by the plug. At 113 days old the plug was removed, thereby restoring a strong (normal) binaural signal. We tested owl 1 immediately and found its localization error had altered dramatically to L $6.7^\circ \pm 4.4^\circ$ and down to $-18.4^\circ \pm 4.1^\circ$. The error not only remained large for the next 4 days, but increased slightly on the third day to L $7.8^\circ \pm 1.7^\circ$ and $-19.7^\circ \pm 4.7^\circ$. On the fifth day, azimuth and elevation errors began to decrease sharply. The azimuth and elevation errors returned to normal after 15 and 28 days, respectively.

The pattern of auditory adjustments exhibited by owl 1 was mirrored by owl 2, in which the right ear was plugged (Fig. 2b). By the first week of testing, at 21 weeks old, owl 2 had adjusted to the monaural occlusion: its error was R $1.1^\circ \pm 4.0^\circ$ and $-1.0^\circ \pm 4.0^\circ$. Just after removing the plug on day 146, its error changed to R $12.4^\circ \pm 4.6^\circ$, and $+7.6^\circ \pm 4.5^\circ$; again, the largest localization errors occurred 2–4 days after the plug had been removed. Azimuth errors declined rapidly after the second day and fell to within normal limits by day 11. Elevation errors peaked on the fourth day and returned to normal after 17 days.

The performance patterns of owl 3 were different from those of the first two birds (Fig. 2c). Owl 3 was 27.5 weeks old and had fully adjusted to the monaural impairment when its right plug was removed: its localization error was L $1.9^\circ \pm 2.5^\circ$ and $-0.8^\circ \pm 4.1^\circ$. After removing the plug its error changed to R $6.9^\circ \pm 3.3^\circ$ and $+6.2^\circ \pm 6.1^\circ$. However, in this case adjustment to the unplugged condition was exceptionally slow: the azimuth error took 25 days to correct and elevation error was still $+3.7^\circ$ after 70 days of normal experience. The difference between owl 3 and the first two birds was the age at plug removal: whereas owl 3 was 27.5 weeks old, owls 2 and 1 were 21 and 16 weeks old, respectively. The data suggest that in the older bird the localization mechanism had become resistant to modification by experience before complete readjustment could occur.

The implication that the localization mechanism becomes less plastic with age is supported by the results from owl 4. The right ear of owl 4 was occluded at 27 weeks of age (188 days) and kept occluded for the next 23 weeks, with no sign of adjustment. Before the plug was removed the owl's error was still L $7.6^\circ \pm 2.1^\circ$ and $-12.4^\circ \pm 4.1^\circ$, and on the day after plug removal its localization accuracy was normal (R $0.7^\circ \pm 2.1^\circ$ and $0.0^\circ \pm 1.8^\circ$). Thus, whereas the owls occluded at 4 weeks of age adjusted completely to the monaural impairment after only 12 weeks, owl 4 did not seem to adjust at all after 23 weeks.

A second set of plugging experiments performed on owls 1 and 2 at 21 and 26 weeks of age, respectively, corroborate the results from owl 4 (Fig. 2). We plugged the right ear of both owls, that is, the opposite ear for owl 1 and the same ear for owl 2 as

were plugged the first time. Neither owl corrected for the induced localization error. Initially the data from owl 1 suggested some adjustment (Fig. 2a), but when the plug was removed after 33 days, the owl immediately localized with normal accuracy ($0.0^\circ \pm 2.7^\circ$ and $+1.7^\circ \pm 2.5^\circ$). Similarly, owl 2, whose ear plug was still in place after 77 days, showed no sign of adjusting its error, which remained at $L 10.8^\circ \pm 2.5^\circ$ and $-9.3^\circ \pm 3.7^\circ$. The lack of adjustment by owl 2 is particularly revealing because the induced hearing impairment was the same in the first and second experiments (right plug). The owl corrected its error the first time, but not the second. Hence the processes responsible for the adjustment in the first plugging experiment were inoperative in the second, again suggesting that plasticity in the localization mechanism disappears or decreases significantly with age.

During normal growth and development, this capacity for adjustment enables the owl to fine-tune its localization mechanism during the period in life when binaural cues are changing rapidly. After the owl reaches adult size and these cues stabilize, the calibration of the localization mechanism begins to crystallize and finally becomes resistant, if not immune, to further modification by experience.

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Defective T-cell response in beige mutant mice

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A role for T cells in immune surveillance has long been suggested¹, but the lack of high tumour incidence in T cell-deficient nude mice represented a challenge to this hypothesis. The discovery of natural killer (NK) cells, which can lyse tumour cells without any previous sensitization, and high NK levels in nude mice, suggested that these cells may constitute a first line of defence against spontaneously arising tumours *in vivo*². Reports³⁻⁵ of a selective NK deficiency and normal T-cell function in C57BL/6 mice carrying the beige mutation (*bg/bg*) suggested that these mice might be useful for assessing the relative importance of T-cell and NK-cell systems in immune surveillance. However we report here that the deficiency of beige mice is not restricted to NK cells. The generation of cytotoxic T lymphocytes (CTLs) in response to alloimmune challenge *in vivo* or *in vitro* was markedly impaired in beige mutants. Thus our results do not support the suggestion^{3,5} that beige mice might be useful as a model of a selective NK deficiency.

To examine the generation of alloimmune CTLs in beige mice, 50 mutant (*bg/bg*) and heterozygous control (*bg/+*) mice ($H-2^b$) were immunized with a single intraperitoneal (i.p.) injection of P815 ($H-2^d$) tumour cells. Five mice from each group were killed at different time intervals after immunization and the anti-P815 cytotoxic activities of individual spleen cell preparations were determined at various effector/target ratios in a 4-h ^{51}Cr release assay⁶. Lytic units (LU) of alloimmune CTL activity were determined from the dose-response curves⁷. The P815 subline used in these experiments was not significantly susceptible to lysis by mouse NK cells, target lysis being 0.8-2.8% (effector/target = 100:1) by normal spleen cells from unimmunized mice and 2.5-5.4% by interferon-activated mouse spleen cells⁷. Results (see Fig. 1) indicate that the CTL activity in *bg/+* mice increased rapidly from day 5 and reached a peak value of 33 LU per 10^6 spleen cells on day 11. CTL response was specific to P815 target cells and was not observed against syngeneic EL-4 cells (results not shown). By day 17, the activity had declined considerably but a secondary challenge *in vivo* with 2.5×10^6 P815 cells increased the level of CTLs in *bg/+* mice observed 1 week after the second challenge. At all time points, anti-P815 CTL reactivity was markedly lower in *bg/bg* mice; the peak level of CTLs on day 11 was 11 LU per 10^6 spleen cells. Moreover, a secondary immunization did not increase CTL activity in *bg/bg* spleens. Lytic units in individual spleen cell suspensions indicated considerable variation between animals (Table 1). However, analysis of variance showed that the difference in generation of CTL activity

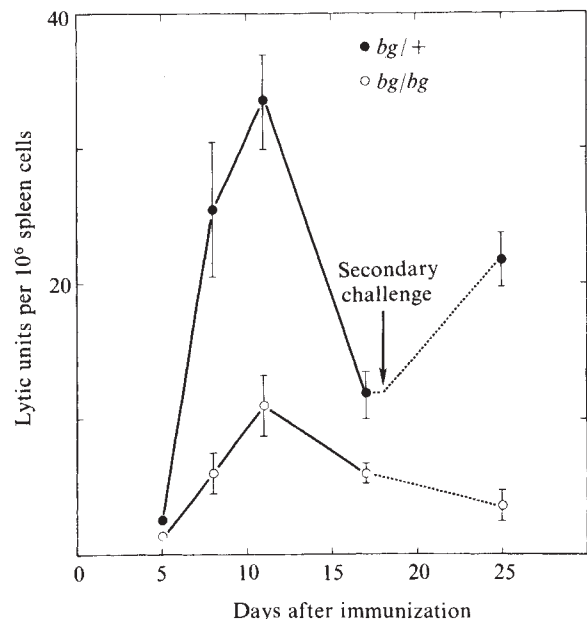


Fig. 1 On day 0, 6-week-old female *bg/bg* or *bg/+* mice were immunized by i.p. injection of 0.2 ml of a P815 tumour cell suspension containing 1×10^7 cells. Tissue-cultured P815 cells used for immunization were washed five times with normal saline before use. On days 5, 8, 11 and 17, five mice each from *bg/bg* and *bg/+* groups were killed and the anti-P815 immune T-cell reactivities of individual spleen cell preparations tested in a 4-h ^{51}Cr release assay at effector/target ratios of 100:1, 50:1, 25:1, 12:1, 12:1 and 6:1 as described previously⁶. Lytic units were calculated from the linear portion of semilog dose-response curves⁷. Each lytic unit represents a target cell lysis of 10% in the test system. Each point in the curve represents the mean $\pm$ s.e.m. of lytic units obtained from five spleen cell preparations. On day 18 mice were reimmunized with another i.p. dose of P815 tumour cells (2.5×10^6 cells in 0.1 ml saline) and spleen T-cell cytotoxicity was examined on day 25. Spleen cells from unimmunized control mice had no detectable anti-P815 cytotoxic activity.

Table 1 Analysis of variance for data in Fig. 1

Lytic units per 10 ⁶ spleen cells*					
Mice	Day 5	Day 8	Day 11	Day 17	Day 25
<i>bg/+</i>	1.86	21.68	32.48	15.44	25.65
	2.46	9.28	38.16	12.48	14.98
	2.32	21.92	36.00	15.12	18.20
	2.68	31.76	41.12	6.64	22.86
	3.56	42.08	20.88	9.68	25.22
<i>bg/bg</i>	1.14	6.28	13.04	2.80	1.69
	1.46	1.36	15.20	4.20	6.46
	0.98	9.96	20.88	6.88	4.28
	1.78	8.56	10.64	5.92	1.43
	1.28	3.86	7.28	5.88	3.87
Source of variation	d.f.	Sum of squares	Mean squares	<i>F</i>	
Days after immunization	4	2,597.8	649.5	49.4 (<i>P</i> < 0.001)	
Beige mutation	1	2,144.2	2,144.2		
Error (includes animal-to-animal variation)	44	1,911.3	43.4		
Total	49	6,653.3			

* Each lytic unit represents 10% target lysis in the assay system and was computed from the linear parts of individual dose-response curves as described elsewhere⁷.

between *bg/bg* and control mice was highly significant (*P* < 0.001; Table 1).

Administration of tumour cells may induce two types of lymphocyte-mediated cytotoxic activity in mice. An early augmentation of spleen NK activity peaks on the third day after tumour administration and falls rapidly thereafter to baseline levels by day 6 or 7 (ref. 8). A second peak of specific alloimmune CTL activity occurs later, between days 10 and 11 after immunization⁹. In our experiments, anti-P815 cytotoxic activity of spleen cells was first determined 5 days after immunization and the peak of cytotoxic activity occurred on day 11—a typical CTL response. Treatment of spleen cells with monoclonal anti-Thy-1 antibodies and complement completely abolished their cytotoxicity for P815 target cells, confirming the T-cell nature of the effector cells (results not shown). The monoclonal anti-Thy-

Table 2 Generation of cytotoxic T cells in a mixed lymphocyte reaction by *bg/bg* or *+/+* mouse spleen responder cells

Source of spleen cells	Anti-P815 cytotoxicity generated in mixed lymphocyte culture (I.U. per 10 ⁶ spleen cells)
<i>+/+</i>	132.4 ± 13.7
<i>bg/bg</i>	39.2 ± 5.4 (<i>P</i> < 0.001)

Responder spleen cells were derived from 11-week-old female *bg/bg* or *+/+* mice (C57BL/6). In each experiment, 50 × 10⁶ responder cells were incubated with 10 × 10⁶ X-ray irradiated (1,000 rad) DBA/2 (12-week-old, female) spleen cells for 5 days in conditions described elsewhere⁵. At the end of incubation, cells were washed and tested for cytotoxicity against ⁵¹Cr-labelled P815 cells in a 3-h ⁵¹Cr release assay at effector/target ratios of 100:1, 50:1, 25:1, 12:1, 6:1 and 3:1. Lytic units were calculated from the linear portion of the dose-response curve as described elsewhere⁷. No significant cytotoxicity was detected in cultures without DBA/2 stimulator cells. Each value is the mean ± s.e.m. of four experiments.

1 antibody used has previously been shown to abrogate completely immune T-cell reactivity with no significant effect on the levels of anti-YAC NK activity in spleen cells¹⁰.

Generation of alloimmune CTLs was also studied in a mixed lymphocyte culture system using a method described elsewhere⁵, except that, instead of mitomycin treatment, the DBA/2 stimulator spleen cells were X-ray irradiated (1,000 rad). Results of four experiments (summarized in Table 2) indicate that the levels of cytotoxicity generated by *bg/bg* spleen cells in 5-day MLC were only 30% of control levels. Cytotoxic activity generated in MLC experiments could also be totally abrogated by treatment with anti-Thy-1 + complement (results not shown).

Thus, we could not confirm the presence of a normal T-cell function in *bg/bg* mice; the reason for the discrepancy between our data and those of Roder³⁻⁵ is unclear. The source of *bg/bg* and control mice (Jackson Laboratories) was the same for both studies. A possible explanation is the considerable variation between animals in levels of alloimmune T cells generated in some beige mice, which can approach the lower limits of a normal response (Table 1). This variation was not reported in the previous study³⁻⁵ and could have contributed to the apparent normality of T-cell function observed in *bg/bg* mice; in addition, cells from different mice were pooled and the immune T-cell activity of the pooled spleen cells determined. In certain cases the results of assays using pooled spleen cells may not represent the mean of activities of individual spleen cell preparations¹¹. Finally, the data presented by Roder *et al.*³⁻⁵ did indicate a lower than normal level of concanavalin A-induced T cell-dependent cytotoxicity in *bg/bg* spleen cells (Figs 6, 8 of ref. 5).

To compare the magnitude of the CTL induction defect in *bg/bg* mice with the NK cell defect, we attempted to quantify the NK activity in spleen cell preparations derived from *bg/bg* and *bg/+* mice. Anti-YAC NK lytic units per 10⁶ spleen cells in *bg/+* spleen cell preparations were 6.26 ± 1.0 (mean ± s.e.m. of eight mice) compared with <0.5 LU in *bg/bg* mouse spleen cells. These results confirmed the previously reported NK defect in *bg/bg* mice³⁻⁵.

Thus we have demonstrated a marked defect in beige mice in the generation of alloimmune T cells both *in vivo* and *in vitro*. Whether the defect in T-cell response in *bg/bg* mice is generalized is unknown. Significantly lower than normal CTL response to lymphocytic choriomeningitis virus infection has been demonstrated in *bg/bg* mice¹², whereas the response to vesicular stomatitis virus was approximately normal¹³. It is therefore possible that a normal or a subnormal CTL response in *bg/bg* mice may depend on the antigen used. Our preliminary findings stress the need for caution when using *bg/bg* mice as a model for a selective NK deficiency with normal T-cell function. Small but significant differences in resistance to certain leukaemias between *bg/bg* and *bg/+* mice¹⁴ could result from a NK-cell defect or T-cell defect or both.

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Further studies on supposed lamarckian inheritance of immunological tolerance

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We have previously reported¹ an unsuccessful attempt to confirm the claim of Gorczynski and Steele^{2,3} that tolerance to alloantigens, induced by the inoculation of F₁ hybrid spleen and bone marrow cells in newborn mice, can be transmitted by males to their offspring. Our study had been designed to reproduce the *in vitro* data of Gorczynski and Steele and to extend them by the transplantation of F₁ hybrid skin allografts. It followed that of Gorczynski and Steele² very closely but differed from it in two respects: the number of semi-allogeneic lymphoid cells in the inoculum used to establish tolerance in the fathers was 5×10^7 instead of 10^8 and the ratio of spleen cells to bone marrow cells was 9:1 rather than 1:1. Although we argued¹ that it is improbable that these differences of execution could have significantly affected the outcome of the experiments, our departure from the original protocol was nevertheless open to criticism⁴ and the purpose of the present further study was to repeat the experiments by following Gorczynski and Steele² in every respect. We also report an attempt to confirm the work of Guttman's group^{5,6}, who claimed that a single CBA male made tolerant to A/J histocompatibility antigens, and mated with normal (CBA $\times$ A)F₁ females, produced offspring with a higher incidence of hypo- or unresponsiveness to A/J skin or tumour allografts than the offspring derived from normal males. We have again failed to confirm the work of Gorczynski and Steele and, like Steinmuller⁷, we have been equally unsuccessful in confirming the observations of Guttman *et al.*

Five newborn CBA/Ca (H-2^a) males from two litters were made tolerant to A/J (H-2^b) histocompatibility antigens by the intraperitoneal (i.p.) inoculation of 10^8 adult male (CBA $\times$ A)F₁ hybrid spleen and bone marrow cells, mixed at a ratio of 1:1. These mice received 5×10^7 cells of the same cell mixture i.p. every 2 weeks throughout the breeding programme. Strain A tail skin allografts were transplanted to them soon after completion of breeding and 4 weeks after their splenic cells (obtained by partial splenectomy) had been tested in the cell-mediated lympholysis assay (CML). The mice were then ~4 months old; all accepted their grafts and were clearly fully tolerant whereas the six normal age-matched control fathers rejected their grafts in the expected manner at ~11 days.

Both tolerant and normal males were mated with groups of three normal CBA females of the same age and, when these had become pregnant, with a further group. Of the five tolerant males, one was sterile, as was one of the six controls. A total of 87 offspring from tolerant fathers ('experimental progeny') and 94 from normal fathers ('control progeny') were raised. Splenic CML data were obtained for 81 (93%) experimental and 79 (84%) control mice and all, with the exception of 4 experimental and 3 control mice that died as a result of partial splenectomy or after skin transplantation, were assessed for reactivity to (CBA $\times$ A)F₁ hybrid skin allografts. Skin grafting and assessment of graft survival, splenectomy, the splenic CML technique and the statistical analysis were exactly as described previously¹.

All spleen suspensions were tested for their ability to respond following mixed lymphocyte culture not only against A antigens but also against 'third-party' C57BI/10 antigens. The latter

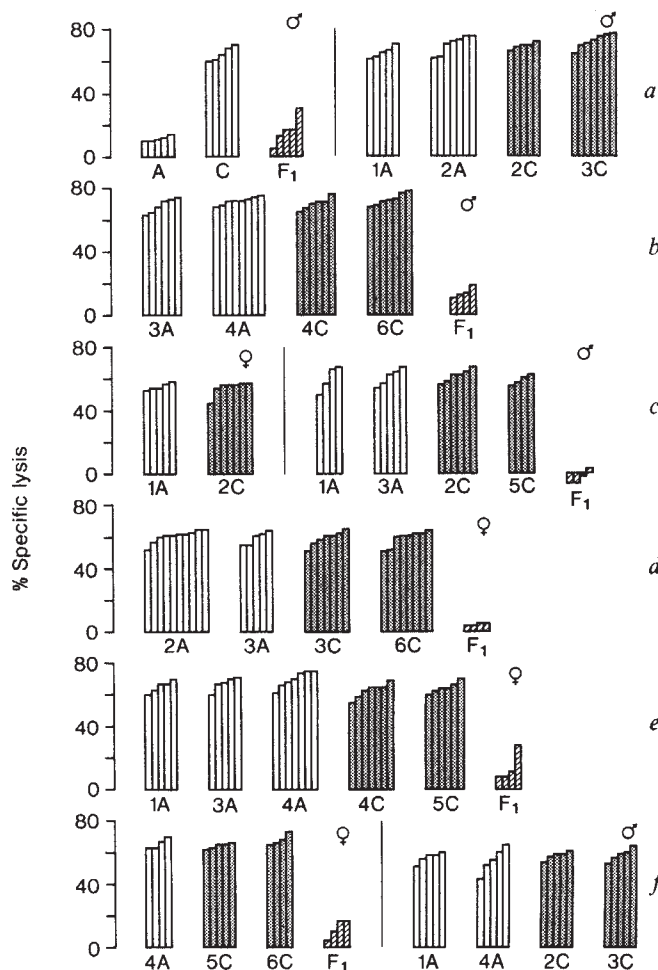


Fig. 1 Anti-A cytotoxic responses of spleen cells from tolerant fathers (A), normal control fathers (C), (CBA $\times$ A)F₁ hybrid mice (F₁) and progeny which are identified by the number of the father followed by A or C. Each panel represents the data from a separate experiment; the sex of the responders is indicated. The height of the bars, which represent responses of individual animals, is the % specific lysis at a killer/target ratio (K/T) of 5:1 derived from a 12-point log regression analysis. Groups 1A and 2C included in a were retested as shown in f (see text). Conditions of culture¹: 4-ml Linbro wells were set up with 10^7 responder spleen cells and 10^7 irradiated (2,000 rad) strain A stimulator spleen cells per well in bicarbonate-buffered RPMI with 5% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, penicillin and streptomycin, glutamine and 10 mM HEPES. The assay was carried out on day 5 of culture. Cells were collected from wells, counted and serially diluted (two-fold) in round-bottomed 0.2-ml microtitre wells to which were added a constant number of ⁵¹Cr-labelled target cells (24-h concanavalin A blast cells) to give K/T ratios starting at ~10:1. Supernatants were collected after 3 h incubation. Per cent specific lysis was taken as $(E-C)/(M-C) \times 100$, where E was the c.p.m. for the experimental supernatant, C the c.p.m. for the medium control supernatant and M the c.p.m. of target cells lysed with 5% Triton.

acted as a positive control to safeguard against technical failure and in all cases a good response was obtained; they are therefore not included in Fig. 1, which illustrates CML reactivity against the specific A antigens only. This time we confined ourselves entirely to the use of spleen cells for *in vitro* testing, as Gorczynski and Steele² had done in their original study, instead of using peripheral blood lymphocytes as well as spleen cells. Two changes were made to our previous protocol: first, the fathers were tested for CML reactivity at 4 months instead of 10 months and, second, (CBA $\times$ A)F₁ hybrid control mice were included in each of the major experiments to provide a baseline

Table 1 Survival of (CBA×A)_F₁ hybrid skin allografts transplanted to the progeny of tolerant or control CBA males mated with normal CBA females

		Graft survival time (days)															
Father	Sex	Experimental progeny								Control progeny							
1	♂	<11	<11	<11	<11	<11	<11	15	15	NA							
	♀	<11	<11	<11	<11	<11	<11	<11	<11								
2	♂	<11	<11	<11	<11	<11	<11	<13	15	<11	<11	<11	<11	<11	<11	15	16
	♀	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	12	14	
		<11	<11	<11	<11												
3	♂	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	
		13	13							12	12	13	13	14	16	16	
	♀	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	13		
4	♂	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	12	12	13	14		
		12	13	13	15	15	15										
	♀	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	<11	13	13		
5	♂	NA								<11	<11	12	17				
	♀									<11	<11	<11	<11	<11	<11	<11	<11
6	♂	NA								<11	<11	<11	<11	<11	12	13	15
										<11	<11	<11	<11	<11	<11	<11	<11
	♀									<11	<11	12	15				
Totals	♂	43								45							
	♀	40								46							
Median survival times		<11								<11							

The sex of the graft donors and recipients was the same. NA, not applicable.

for the CML data. Both changes must be regarded as improvements.

Figure 1a shows that the experimental fathers (A) were indeed fully tolerant, thus confirming the skin graft data. Not only do their responses show a highly significant difference when compared with the controls (C), but it is clear that they did not exceed the baseline provided by the F₁ controls. The experimental males were, therefore, as tolerant as the F₁ controls. The low level of cytotoxicity in the F₁ mice is in agreement with the F₁ anti-parental cytotoxic responses described for other strain combinations⁸. Because the tolerant fathers would be expected to be chimaeric for F₁ hybrid lymphoid cells it is possible that the presence of F₁ cells contributed to the low level of responsiveness shown by the tolerant fathers.

As for the progeny, Fig. 1a-f indicates that there were no differences of any interest between experimental and control progeny. The differences between the control progeny and the F₁ mice were always highly significant ($P = 0.007-0.003$). Only two experimental groups deserve special mention. First, group 1A (Fig. 1a) showed a borderline hyporesponsiveness compared with the male controls ($P = 0.04$), but a repeat

experiment some weeks later did not confirm this (included in Fig. 1f). Second, group 4A (Fig. 1e) was marginally hyper-responsive compared with all controls ($P = 0.01$) and there was a slight hyper-responsiveness when all experimental and control mice of panel e were compared ($P = 0.02$). No importance whatever is attached to this, especially as the anti-C57BI/10 response by this group was also slightly greater than that of the controls ($P = 0.01$). The conclusion is inescapable that the experimental progeny did not display the hypo- or non-responsiveness described by Gorczynski and Steele.

The skin graft data again fully support this. Table 1 lists the survival times of individual (CBA×A)_F₁ hybrid grafts in experimental and control offspring. It will be seen that they do not differ, and this is borne out by statistical analysis (males, $P > 0.3$; females, $P > 0.2$). Because F₁ grafts were used, a few were rejected a little more slowly than the majority, especially by male recipients, but this applied equally to the experimental and control progeny.

We have therefore confirmed our previous study and failed to substantiate the claim that acquired immunological tolerance can be transmitted to offspring by tolerant male mice. Other workers have experienced similar difficulties in a variety of models involving immunological tolerance, from neonatally induced tolerance⁹⁻¹¹, the tolerance of tetraparental^{12,13} or adult parabiont¹³ mice, to the natural tolerance that characterizes F₁ hybrids¹⁴.

We are again unable to explain the discrepancy between our latest negative results and the original findings of Gorczynski and Steele. Our study was, as before, conducted 'double-blind' in that those of us performing the *in vitro* test were presented with the splenic biopsies in coded form and the code was not broken until the data had been fully computed. Care was taken to ensure that both skin grafting and graft assessment was carried out without bias. We feel we have gone as far as is possible to repeat the precise conditions of the original study as reported by Gorczynski and Steele² and we must conclude that the phenomenon described by them was due to some special conditions prevailing in their laboratory. Although that might still be of some interest it is unlikely that the Lamarckian inheritance they postulate is of any importance in the evolutionary process, if indeed it exists.

We have also failed in our attempt to confirm the observation by Guttman's group^{5,6} that the offspring produced from a mating between a C3H male, made tolerant at birth by the inoculation of strain A spleen cells, and normal (C3H×A)_F₁ hybrid females, accepted A strain skin allografts more readily than controls. Two kinds of functionally tolerant males were used in our

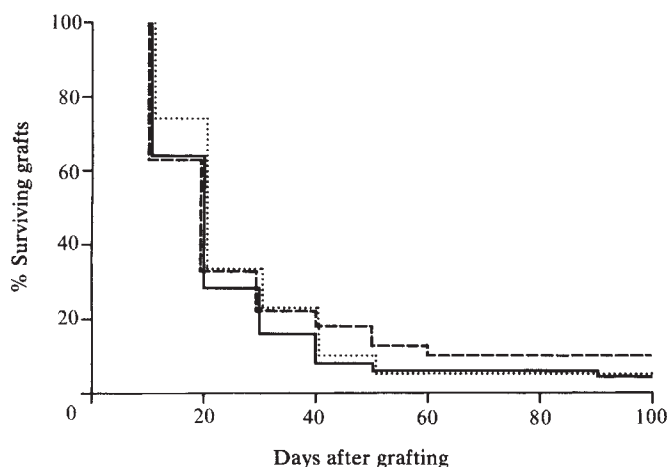


Fig. 2 Survival of female (CBA×A)_F₁ tail skin grafts transplanted to the female progeny of tolerant and normal control CBA males mated with normal (CBA×A)_F₁ females. — Tolerant; fathers received F₁ lymphoid cells at birth and every 2 weeks thereafter (79 mice); tolerant; fathers received F₁ cells at birth only (39 mice); --- normal fathers (40 mice).

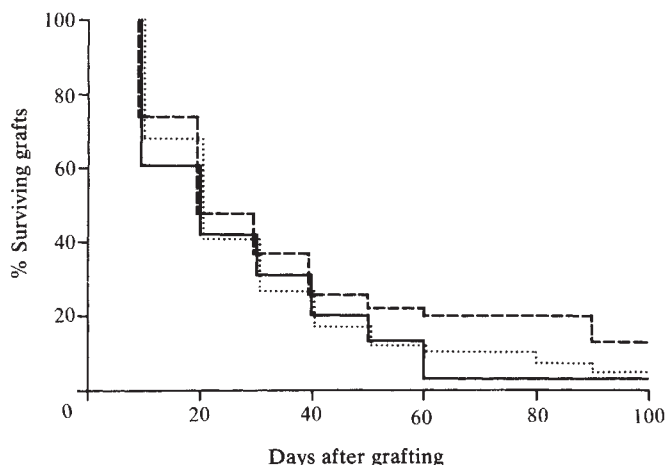


Fig. 3 Survival of male (CBA $\times$ A) F_1 tail skin grafts transplanted to the male progeny of tolerant and normal control CBA males mated with normal (CBA $\times$ A) F_1 females. — Tolerant; fathers received F_1 lymphoid cells at birth and every 2 weeks thereafter (64 mice); tolerant; fathers received F_1 cells at birth only (41 mice); --- normal fathers (46 mice).

investigation: 10 that had received 5×10^7 spleen and bone marrow cells at birth (in a ratio of 9:1) and the same number every 2 weeks until completion of the breeding programme (designated A), and 4 that had been injected at birth only (designated B). Controls (designated C) were provided by normal age-matched males. All offspring were grafted with (CBA $\times$ A) F_1 skin 2–3 months after birth. The only ways in which our protocol differed (because it was carried out during another study¹) were in the use of CBA mice in place of C3H, although these are compatible for H-2 antigens and similar to C3H mice, and in the use of a mixture of spleen and bone marrow cells. The mice were grafted in five batches over several months, with all groups represented in most batches, and it was sometimes difficult to assess with complete accuracy the end point of survival for grafts that had undergone a slow, insidious form of rejection.

Figures 2 and 3 show that there was no substantial difference between the progeny of groups A, B and C when comparing males with males and females with females. There is no statistically significant difference in the numbers surviving beyond 100 days between any of the groups. If the evaluation is made at 50 days, only the comparison between the male progeny of groups B and C shows a marginal but not significant difference, though in an unexpected direction in that the number of experimental B progeny with surviving grafts is lower than in the control group, using a one-tailed test (χ^2 2 $\times$ 2 test with Yates' correction for continuity; $P < 0.05$). Like Steinmuller⁷ we are therefore unable to confirm the claim by Guttman and his colleagues in the strain combination used by us.

All advocates of Lamarckism have insisted that the only valid test of whether or not it occurs is that based on the inheritance of specific adaptive changes acquired during an organism's lifetime. The immune response and its specific diminishment (tolerance) are admirably well suited to make the basis of a test for Lamarckism. In their original paper on tolerance, Billingham *et al.*¹⁵ (see also ref. 9) found that the progeny of CBA parents both tolerant to A strain grafts reacted normally to such grafts, but they might have been mistaken because it was not at that time possible to measure alloimmunity as precisely as may be done today. The results reported here indicate that they were probably right and that in this experimental system acquired tolerance is not inherited.

All examples of supposedly lamarckian inheritance proposed so far¹⁶ have been mistaken or open to more plausible explanations, especially in terms of selection. On the basis of our new evidence we can say with confidence that in respect of the

systems we have been investigating, lamarckian inheritance does not obtain.

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Adult haematopoietic cells transplanted to sheep fetuses continue to produce adult globins

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In man, several primates, sheep and goats, fetal haemoglobin (Hb F: $\alpha_2\gamma_2$) is synthesized during the intra-uterine life, with a switch to adult haemoglobin (Hb A: $\alpha_2\beta_2$) production around birth. The mechanisms underlying the switches in globin gene expression are unknown. Studies of erythroid cell cultures suggest that haemoglobin switching is controlled at the level of haematopoietic progenitor cells^{1,2}. To test whether interactions between the haematopoietic environment and the haematopoietic progenitor cells have a role in the haemoglobin switch, we transplanted adult cells into unrelated sheep fetuses at a time of gestation when only fetal haemoglobin was being produced in the recipient cells. We report here that there was engraftment of the donor cells and bone marrow chimaeras were formed. The donor cells, proliferating and differentiating in the fetal environment, continued to produce adult haemoglobin. These results suggest that the fetal environment does not contain elements capable of switching the adult haematopoietic cells to a fetal programme of haemoglobin synthesis.

In the sheep, gestation lasts ~145 days and the switch from fetal to adult globin formation usually starts at ~120–130 days and is completed shortly afterwards³. Fetuses younger than 110 gestational days typically have 100% fetal haemoglobin in their red cells (by chemical analysis). In the adult sheep there is a genetic polymorphism at the β locus of the adult haemoglobin⁴ with two alleles: β^B , providing chains for Hb B ($\alpha_2\beta_2^B$), and β^A , directing the synthesis of Hb A ($\alpha_2\beta_2^A$). The existence of this genetic polymorphism allowed us to use haematopoietic cell transplantations to determine the regulation *in vivo* of haemoglobin switching⁵ as follows. Bone marrow cells of an adult,

homozygous Hb B donor were transplanted to fetuses produced by AA $\times$ AA crosses (and hence genetically homozygous for Hb A) that were at a gestational age when they produced only fetal haemoglobin. We tested the red cells of the recipient for the presence of Hb B, to obtain information about (1) the fate of the transplanted cells, that is, whether or not, there was engraftment and (2) the globin synthesized by the cells after transplantation to the fetal environment. For example, if after transplantation, there was no production of donor Hb B in fetal red blood cells but a chimaeric A/B sheep was born we could deduce that the donor cells were engrafted but had switched during fetal life to fetal globin formation. Synthesis of Hb B during gestation and the birth of a chimaeric A/B animal would be evidence that the donor cells were engrafted but did not switch to fetal haemoglobin formation.

We used pregnant ewes having confirmed dates of gestation. Two heparin-filled polyvinyl catheters (0.9 mm o.d.) were inserted into the femoral artery and vein of the fetus. Direct access to the fetal circulation was gained through a single midline incision of maternal skin and body wall, incision of the uterus, delivery of the fetal hind leg and placement of one catheter each into the fetal femoral artery and vein. All incisions were then closed around the catheters, which were secured to the maternal skin; 48 h after surgery, each fetus received the donor bone marrow cells (4×10^8 nucleated cells per estimated kg fetal body weight), via the venous catheter, which was removed immediately after the bone marrow infusion. Fetal blood samples were obtained via the arterial catheter for haemoglobin analysis before and at various intervals after infusion. To minimize the risk of infection, the catheter was removed 3 weeks after the transplantation and the pregnancy allowed to go to term. Blood sampling was resumed in the newborn in the case of successful deliveries.

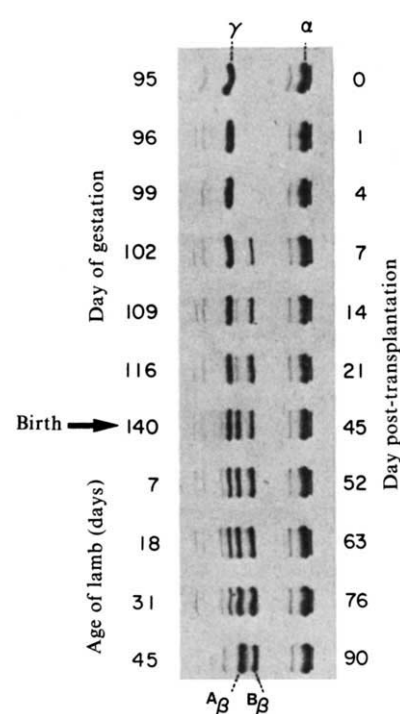
All the fetuses were at day 85–105 of gestation. As the donors and recipients were unrelated, in the initial studies we attempted to suppress the haematopoiesis of the recipient fetus before transplantation. The fetuses were irradiated or immunosuppressed but all the recipients died within 1–6 days after these manipulations. Subsequently, we simply transfused the adult bone marrow cells into untreated fetuses. Eleven AA fetuses were given bone marrow cells from the same unrelated female BB donor. Six of these were aborted after this treatment; as autopsies were not done, the reason for these losses is unknown. The haemoglobins of the five surviving fetuses were analysed by globin chain isoelectric focusing. No β^B (donor) or β^A (recipient) globin chains were detected in any of the fetuses on days 1 and 4 post-transplantation, indicating that the minor contamination of the donor's buffy coat cells by red cells did not influence the haemoglobin phenotype of the fetus. Two fetuses which were given infusions, respectively, on days 92 and 105 of gestation, failed to exhibit β^B chains (donor) both throughout the 3 weeks of post-transplantation intra-uterine blood-sampling and during postnatal sampling (up to 41 and 53 days, respectively, after birth).

Three fetuses were chimaeric A/B at birth. One was studied only at birth and postnatally; a problem with the catheters did not allow intra-uterine sampling in this case. This animal remained healthy and was A/B chimaeric throughout the period of sampling. Two of the chimaeric fetuses were sampled *in utero* for 21 days post-transplantation. One of them died after birth, but the second developed into a healthy animal which remained chimaeric. Figures 1 and 2 show the results of haemoglobin analyses. The adult haemoglobin of the donor was clearly detectable by day 7 post-transplantation and comprised 41% of the fetal haemoglobin by day 21 post-transplantation.

The two living chimaeric lambs (a male and a female) produced both Hb B and Hb A at a ratio of 40:60. As the bone marrow donor was female, chromosomal studies were made of cells from the sole male chimaeric survivor. Both donor (XX) and recipient (XY) karyotypes were noted for 50 cells; 58% of the lymphocytes were of the donor (XX) karyotype.

Fig. 1 Globin-chain isoelectric focusing of lysates of blood samples obtained from a fetus pre- and post-transplantation. Note the absence of detectable β -chains before transplantation and on days 1 and 4 post-transplantation. The donor's β^B chains were detectable by day 7 and the intensity of the β^B zone increased thereafter. Note also the appearance of significant amounts of β^A chains at birth and that the animal remained β^B/β^A chimaeric. With the globin-chain isoelectric focusing method used⁶, we achieved clear separation of the globin subunits. Subunit β^C (from the inducible haemoglobin C: $\alpha_2\beta^C_2$) migrates between subunits β^A and β^B . We did not observe β^C -chain synthesis during the intra-uterine sampling of the animals with or without evidence of engraftment. The AA fetuses were given cells from the same 2.4-yr old female donor (see text).

Donor bone marrow cells were obtained by multiple aspirations from different sites on the posterior iliac crest. The heparinized bone marrow samples were filtered through a screen mesh to remove large particles; the filtrate was allowed to settle at room temperature for 15 min and the supernatant and buffy-coat layers were removed and passed through needles of progressively smaller pore sizes to achieve a single-cell suspension. The cells were resuspended in Hanks' balanced salt solution containing the recipient's own plasma (5% v/v) at a concentration of $4\text{--}7 \times 10^7$ nucleated cells ml^{-1} . In all cases the cells were infused into the fetus within 1–2 h of aspiration.



These results raise questions for cell immunology and transplantation biology. Successful grafts were observed in three of the five animals surviving to birth and all the donor-recipient pairs were unrelated. The data suggest that in adult fetus transplantation, conditions may exist which both allow tolerance of the donor cells and diminish the possibility of severe graft-versus-host disease. Confirmation of the findings of our experiments and similar studies in primates may have practical relevance. If untreated primate fetuses are infused with bone marrow cells from unrelated donors a chimaeric primate may be formed. Thus, intra-uterine transplantation of adult bone marrow cells could be considered as an alternative to abortion, when, on prenatal diagnosis, a human fetus is found to be homozygous for a β -chain haemoglobinopathy (such as β -thalassaemia or a Hb S syndrome).

The cellular mechanisms involved in the haemoglobin switch during ontogeny are unknown. The question remains as to whether the switch from fetal to adult globin formation is intrinsic to the cell (that is, due to the operation of a developmental clock which informs the cells when or after how many divisions the switch will occur) or whether it is the result of haematopoietic cell-environment interactions. If such interactions are involved, are there specific factors present during fetal life or produced during and after the switch-over period which influence the programme of the progenitor cells?

To test the possibility of environment-cell interactions we have previously transplanted haematopoietic cells from pre-switch sheep fetuses into lethally irradiated adult recipients. We used the A and B polymorphism of the sheep and in one successful experiment, found that the fetal cells switched to adult globin expression when grafted in to the adult environment⁵. There are two possible interpretations of these results: (1) the adult environment influenced the programme of the transplanted haematopoietic cells and accelerated the switching process; (2) the transplanted cells were intrinsically programmed to switch and did so in their new environment. In the

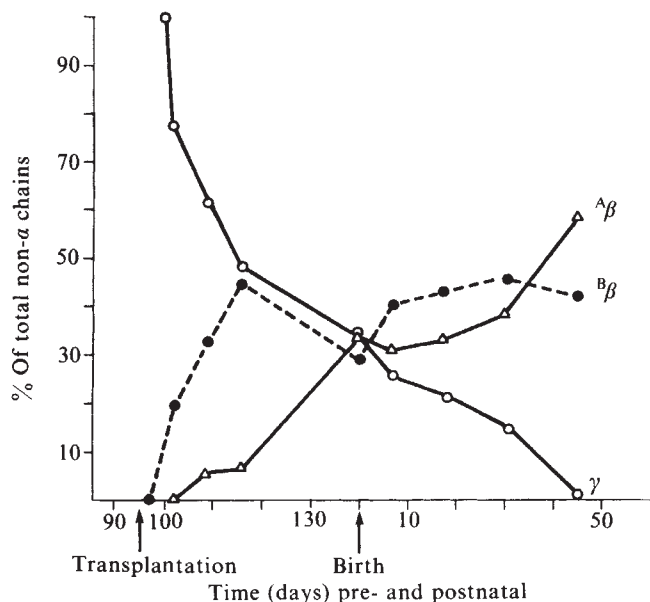


Fig. 2 Proportion of γ , β^A (recipient) and β^B (donor) chains in the haemolysates of blood samples periodically drawn from a fetus (gel shown in Fig. 1). Note the striking prenatal increment in the donor's β^B chains, and the lower proportions of β^B chains at birth compared with the prenatal maximal value. After the $\gamma \rightarrow \beta$ -chain switch was complete, the level of β^A chains (derived from the recipient's cells) exceeded the level of β^B chains (originating from the donor cells). A remarkable aspect of these quantitative analyses is the pre- and post-natal proportion of the donor β^B chain. The findings are compatible with two peaks of β^B synthesis, one ~20 days post-transplantation and the second 20–30 days after birth. A plausible interpretation is as follows. The first peak of β^B chain synthesis derives from red cells which have been formed by transplanted committed erythroid stem cells. These progenitors have amplified, differentiated, been committed to erythroblastic maturation and have provided the fast rising level in donor haemoglobin after infusion of the donor bone marrow cells. The postnatal peak, on the other hand, is derived from red cells produced by the engrafted pluripotent stem cells from the donor.

adult $\rightarrow$ fetal transplantation experiments reported here, we found that the adult donor cells produced large amounts of adult haemoglobin at a time when the cells of the fetuses had not switched to adult haemoglobin synthesis; this suggests that the donor cells continued to follow their adult globin programme even under the influence of the fetal environment. The adult $\rightarrow$ fetal transplantations results are consistent with the mechanisms suggested by the findings of the fetal $\rightarrow$ adult haematopoietic cell transplantations. If the switch from Hb F to Hb A formation is intrinsically controlled, the adult cells did not switch to fetal globin expression because their programme of differentiation had already been intrinsically changed. If environment-cell interactions control the switch, both the fetus $\rightarrow$ adult and adult $\rightarrow$ fetus transplantations point to the establishment of an 'adult' haematopoietic environment as an underlying cause of the changes in haematopoietic cells effecting haemoglobin switching.

Although further experiments are required to assess individually the programmes of the various classes of donor progenitor cells that were transplanted to these fetuses, the data presented here provide evidence against the possibility that there existed at the developmental time of these transplantations, an environment able to switch adult cells to typical fetal haemoglobin synthesis.

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Thrombospondin is the endogenous lectin of human platelets

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Platelets activated by α -thrombin change shape from disks to spheres and aggregate. During this sequence of activation, platelets develop a membrane-bound lectin activity^{1,2} which is expressed after the secretion of intracellular granule contents³. This lectin activity seems to be important in mediating platelet aggregation by binding to a specific receptor on other platelets. Gartner *et al.*⁴ have recently shown that fibrinogen is the receptor for the endogenous lectin secreted by activated platelets. Thrombospondin⁵ (also called glycoprotein G⁶ and thrombin-sensitive protein^{7,8}) is an α -granule constituent which has a molecular weight (MW) of 450,000 and is a disulphide-linked trimer of 160,000 MW subunits^{5,9,10}. Secreted thrombospondin^{5–14} binds to platelet membranes in a calcium-dependent manner¹². We show here that thrombospondin is the endogenous lectin of human platelets.

Thrombospondin and other proteins were tested for lectin activity using minor modifications of the haemagglutination system described by Gartner *et al.*^{1–4}. If thrombospondin is the endogenous lectin of platelets, it may act directly as a haemagglutinin. Because thrombin-treated but not native platelets have lectin activity^{2,4}, we used fixed (formaldehyde-treated) native platelets and fixed thrombin-treated platelets as controls in the haemagglutination assay. As expected, fixed native platelets had no lectin activity (Table 1) whereas fixed thrombin-treated platelets had lectin activity and agglutinated fixed trypsin-treated sheep erythrocytes at a dilution of 1:16 (a titre of 16). This haemagglutination was blocked by α -D-mannosamine or α -D-glucosamine but not by N-acetylmannosamine or N-acetylglucosamine. These same inhibitors have previously been shown to block both platelet plasma membrane lectin activity detected using bovine erythrocytes¹ and thrombin-induced platelet agglutination⁴. When tested in the same system, thrombospondin purified from human platelets had strong lectin activity (haemagglutinin titre of 256, Table 1). This lectin activity was blocked by glucosamine and mannosamine but not by N-acetylglucosamine or N-acetylmannosamine. In contrast, purified human serum albumin, fibronectin, factor VIII, fibrinogen and plasminogen tested as controls had no lectin activity.

If thrombospondin is the endogenous platelet lectin, excess exogenous thrombospondin may also inhibit the haemagglutinating activity of thrombin-treated platelets by blocking thrombospondin receptors. To test this hypothesis, proteins were serially diluted, sheep erythrocytes and fixed thrombin-treated platelets then added and haemagglutination was assessed. A buffer control did not block haemagglutination (Table 2)

Table 1 Lectin activity of thrombospondin

Diluted material	Titre
Fixed native platelets	0
Fixed thrombin-treated platelets	16*
Thrombospondin	256*
Human serum albumin	0
Fibronectin	0
Factor VIII	0
Fibrinogen	0
Plasminogen	0

Haemagglutination of sheep erythrocytes was assayed in microtitre 'V' plates (Cooke Engineering Laboratory Products) as described by Gartner *et al.*¹⁻⁴. 25 μ l of Tris-buffered saline containing calcium (TCS buffer: 10 mM Tris-HCl, 2 mM CaCl_2 , 150 mM NaCl, pH 7.4) was added to each well, then 25 μ l of the diluted material was added to the first well in a row of 12 wells and was serially diluted twice in duplicate series of 11 wells. The remaining well in each series contained no diluted material and served as control. Finally, 25 μ l of a 2.5% suspension of trypsin-treated, formalinized sheep erythrocytes in TCS buffer prepared as described by Gartner *et al.*¹⁻⁴ was added to each well. The wells were agitated gently and evaluated for haemagglutination after standing overnight at room temperature. The titre of diluted material is defined as the reciprocal of the end point dilution (greatest dilution that gives agglutination). Fixed thrombin-treated platelets were prepared as follows. The platelets in 200 ml of fresh platelet-rich plasma were pelleted, washed once with 120 mM KCl, 75 mM Tris-HCl, 12 mM sodium citrate, pH 6.3, twice with 150 mM NaCl, 10 mM Tris HCl, 1 mM EDTA, pH 7.4 (ETS), and then resuspended in 5 ml ETS. The resuspended platelets were treated for 2 min at room temperature with human α -thrombin (3 units ml^{-1} , a gift of Dr John Fenton II) and washed once with ETS and once with 0.15 M NaCl, 10 mM Tris HCl, pH 7.4 (TS buffer). The resulting pellet was resuspended in 2% paraformaldehyde in phosphate-buffered saline and incubated overnight at 4°C. The fixed platelets were washed four times with phosphate-buffered saline and resuspended in TS buffer at a concentration of 350,000 μl^{-1} and stored at 4°C until use. Fixed native platelets were prepared by mixing fresh platelet-rich plasma with an equal volume of 4% paraformaldehyde in phosphate-buffered saline and incubating overnight at 4°C. These platelets were washed as above, resuspended in TS at a concentration of 350,000 μl^{-1} and stored at 4°C until used. Thrombospondin was prepared from 4 units of platelets by the method of Lawler⁵. The thrombospondin was >95% pure and contained no fibrinogen when analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The human serum albumin was obtained from Behring. The purified peak I human fibrinogen was provided by Dr M. Mosesson³² and was >96% clottable and homogeneous on SDS-PAGE. The human plasma factor VIII was prepared from plasma by methods previously described³³ and did not contain fibronectin or plasminogen when analysed for these proteins by enzyme-linked immunosorbent assay. The purified human plasminogen³⁴ and fibronectin³⁵ were gifts from Dr P. Harpel. The factor VIII, plasminogen and fibronectin were absorbed with insolubilized anti-human fibrinogen and were negative for fibrinogen by an enzyme-linked immunosorbent assay. All proteins were added to the first well as 25 μ l of a solution of 0.2 mg ml^{-1} .

* This agglutination was inhibited by a 57 mM final concentration of either α -D-mannosamine or α -D-glucosamine but not by 57 mM N-acetylmannosamine or N-acetylglucosamine.

whereas thrombospondin blocked haemagglutination with an inhibitory titre of 8 (final concentration of thrombospondin in last inhibited well is 6.3 $\mu\text{g ml}^{-1}$). Human serum albumin and fibronectin inhibited haemagglutination only weakly. Fibrinogen also strongly inhibited haemagglutination with an inhibitory titre of 16 in the presence or absence of calcium. These latter results are at variance with those of Gartner *et al.*⁴, who found that fibrinogen inhibited haemagglutination only in the absence of calcium. The discrepancy may be due to the fact

Table 2 Thrombospondin blocks haemagglutination by fixed, thrombin-treated platelets

Diluted material	Inhibitory titre
Buffer	0
Thrombospondin	8
Human serum albumin	2
Fibronectin	2
Fibrinogen	16

The materials listed were added as 25 μ l of a solution of 0.2 mg ml^{-1} to 25 μ l of TCS buffer and serially diluted as in Table 1. 5 μ l of a 12.5% suspension of trypsin-treated, formalinized sheep erythrocytes and 25 μ l of fixed thrombin-treated platelets diluted 1:4 with TSC buffer (haemagglutination titre of undiluted fixed thrombin-treated platelets was 16; see Table 1) were then added and the microtitre plates agitated, incubated overnight at room temperature, and read. The inhibitory titre of a diluted material is defined as the reciprocal of the greatest dilution which inhibited haemagglutination compared with a control without any diluted material. Other details are as in Table 1 legend.

Table 3 Thrombospondin blocks the agglutination of fixed, thrombin-treated platelets

Diluted material	Inhibitory titre
Buffer	0
Thrombospondin	16
Factor VIII	0
Human serum albumin	1
Fibronectin	2
Plasminogen	2
Fibrinogen	16

The materials listed were serially diluted as in Tables 1 and 2. 25 μ l of fixed thrombin-treated platelets diluted 1:4 with TCS buffer were then added and the microtitre plates agitated, incubated overnight at room temperature and read. The inhibitory titre of a diluted material is defined as the reciprocal of the greatest dilution which inhibited agglutination compared with a control without any diluted material. Other details as in Table 1 legend.

that the platelets used in our studies were fixed in formaldehyde after thrombin treatment.

Fixed thrombin-treated platelets also agglutinate when tested alone in a microtitre assay system (titre of 32). If thrombospondin is the endogenous lectin of platelets, excess exogenous thrombospondin may also inhibit the agglutination of fixed thrombin-treated platelets by blocking thrombospondin receptors. To test this possibility, proteins were serially diluted, fixed thrombin-treated platelets added and agglutination assessed (Table 3). Buffer did not inhibit agglutination whereas thrombospondin strongly inhibited agglutination. Factor VIII, human serum albumin, fibronectin and plasminogen inhibited agglutination only very slightly or not at all. Fibrinogen, however, strongly inhibited agglutination.

Of the proteins tested, only thrombospondin had lectin activity (Table 1), blocked haemagglutination by fixed thrombin-treated platelets (Table 2) and blocked the agglutination of fixed thrombin-treated platelets (Table 3). None of the other platelet granule proteins tested had lectin activity and only fibrinogen blocked haemagglutination or platelet agglutination. Studies of other α -granule proteins have shown that neither β -thromboglobulin nor platelet growth factor has lectin activity¹⁵. Further, although platelet factor 4 was shown to have agglutinating activity¹⁵, it differed from the lectin activity of thrombin-stimulated platelets² or thrombospondin in that it was not blocked by mannosamine or glucosamine and was not trypsin sensitive¹⁵. These observations thus support our conclusion that thrombospondin is the platelet lectin.

The observations that thrombospondin has lectin activity, is a multimer and by itself causes haemagglutination (Table 1) suggest that thrombospondin is a multivalent molecule. The ability of thrombospondin to block haemagglutination and platelet agglutination at low concentrations (6.3 and 3.1 $\mu\text{g ml}^{-1}$, respectively) suggests that thrombospondin binds with high affinity to its receptor. The blocking effect of fibrinogen is compatible with the recent observation of Gartner *et al.*⁴ that fibrinogen is the receptor for the endogenous lectin of platelets. If platelets interact with each other during aggregation via intercellular bridges of thrombospondin anchored to platelet-bound fibrinogen, excess free fibrinogen in an agglutination or haemagglutination assay would react with thrombospondin and block the reaction. The same reasoning applies to the blocking effect of thrombospondin. Our data are also compatible with the recent observation that the platelets from patients with the grey platelet syndrome (which lack α -granules and their constituents, including thrombospondin) are deficient in thrombin, ADP and collagen induced aggregation¹⁶.

Other proteins in platelet granules—for example, factor VIII/von Willebrand factor¹⁷⁻¹⁹, factor Va²⁰⁻²², fibrinogen²³⁻²⁵ and fibronectin²⁶—also have major roles in platelet function. Furthermore, it is interesting that endothelial cells, which synthesize von Willebrand factor^{27,28} and fibronectin²⁹, also synthesize thrombospondin^{30,31}. Although the level of thrombospondin in plasma as measured by radioimmunoassay is quite low (D.F.M. and E.A.J., unpublished results), endothelial cells

in certain conditions may release thrombospondin in a local microenvironment and thus support platelet function.

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A microtubule-associated protein in the mitotic spindle and the interphase nucleus

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Accurate chromosome segregation during mitosis is contingent on the controlled polymerization and function of spindle microtubules. Assembly-promoting polypeptides that co-sediment with microtubules during cycles of polymerization *in vitro* are attractive candidates for regulators of the formation and function of the mitotic apparatus *in vivo*. However, previous work has shown that the major species of microtubule-associated proteins (MAPs) from mammalian brain or tissue culture cells are either not associated with the mitotic spindle¹ or are associated with both mitotic and interphase cytoplasmic microtubules²⁻⁴. We have therefore sought a novel way to identify spindle regulatory factors. In the study reported here, monoclonal antibodies have been prepared against a MAP fraction from HeLa cells. One of the resulting hybridoma clones produces IgG that binds to a polypeptide of molecular weight ~200,000. During cell division this antigen is associated with fibres in the mitotic apparatus, but in interphase it is located primarily in the nucleus. Surprisingly, the antigen is also detectable on the plasma membrane of circulating human erythrocytes. This spindle component is distinct from previously characterized MAPs and may represent a regulatory factor in the assembly and function of the mitotic apparatus.

The molecular factors controlling the assembly and function of the mitotic spindle are largely unknown. Interphase microtubules in lysed cell models display a calcium lability that is modulated by MAPs⁵, suggesting that the polypeptides which co-purify with cytoplasmic microtubules may have important regulatory functions. Other potential regulatory proteins, such as calmodulin⁶ and cyclic nucleotide-dependent kinase⁷, have been localized in the mitotic spindle, but although this suggests several models for spindle regulation, the actual functions of these enzymes during chromosome separation remain speculative.

We have been studying MAPs from cultured cells using the lymphocyte hybridoma methodology to identify specific mitotic

spindle components. Using a crude mixture of HeLa cell MAPs purified by cycles of co-assembly with HeLa tubulin and by ion-exchange chromatography⁸ as initial inoculum, we have screened for spindle antigens by radioimmunoassay and immunofluorescence microscopy. The antibody produced by hybridoma clone 0-2D5.3.1 binds a polypeptide from HeLa cells with an approximate molecular weight of 200,000 (Fig. 1). The 0-2D5 antigen is sensitive to endogenous protease both in purified microtubule protein preparations and in whole-cell homogenates⁹, as are the high-molecular weight MAPs from brain¹⁰. The 0-2D5 antibody preparation produces a distinctive punctate nuclear immunofluorescent staining pattern in interphase human cells and stains mitotic spindle fibres intensely (Fig. 2). The interphase microtubule array stains only faintly (Fig. 2a, b). The fluorescent nuclear dots range in number from

Fig. 1 Immunoprecipitation of HeLa cell MAP. Autoradiograph of ³⁵S-methionine-labelled HeLa cell homogenate (a) and *Staphylococcus aureus* immunoprecipitates¹⁷ using 0-2D5.3.1 antibody (b) and a control monoclonal antibody (c) run on a 7.5% SDS-polyacrylamide gel¹⁸. The 0-2D5 antibody specifically precipitates a protein of molecular weight ~200,000, while there are several additional polypeptides that bind nonspecifically to both immune complexes. Molecular weight markers were fibronectin (230,000), phosphorylase a (94,000), bovine serum albumin (68,000) and ovalbumin (44,000). The MAP fraction used as antigen was prepared by ion-exchange chromatography of four times-cycled HeLa cell microtubule protein as described elsewhere⁸. BALB/c mice were immunized intraperitoneally with 100 µg of SDS-denatured HeLa MAPs emulsified in an equal volume of Freund's complete adjuvant. Twenty-one days later the mice were boosted intravenously with 50 µg of MAPs in saline. After another 4 days splenic lymphocytes were collected and fused with SP2/0 myeloma cells as previously described¹. Hybridomas were screened by a solid-phase radioimmunoassay, and cells producing specific antibody were cloned twice in soft agar. Immunoglobulin from 1.0 l suspension cultures was concentrated 100-fold by 40% ammonium sulphate fractionation and the IgG purified by chromatography on Affi-gel Blue CM (Biorad), followed by another ammonium sulphate precipitation.

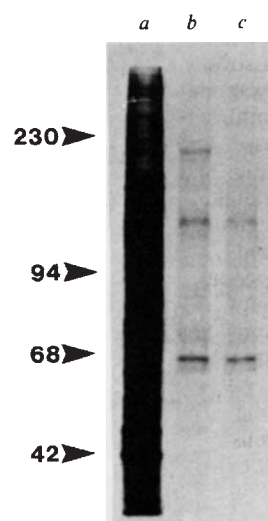
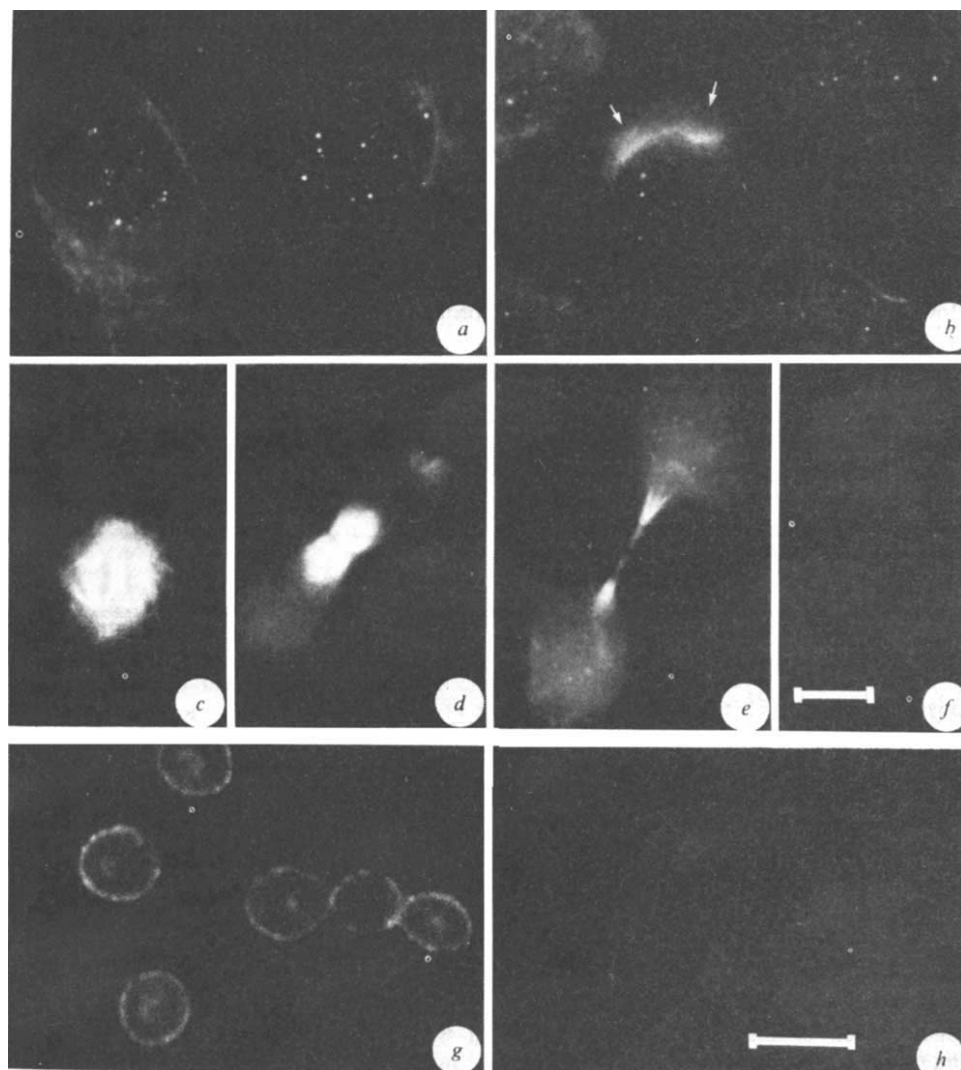


Fig. 2 Indirect immunofluorescent staining pattern of HeLa (*a, b, d-f*) and Va90 (*c*) cells and human erythrocytes (*g, h*). Cells were stained with anti-MAP antibody secreted by hybridoma 0-2D5.3.1 (*a-e, g*) or with a control mouse monoclonal preparation (*f, h*). The 0-2D5 antibody stained the interphase nucleus (*a, b*) and the enlarging prophase asters (*b*, arrows) in human cells. The mitotic spindle stained intensely (*c-e*) with the punctate pattern reappearing during telophase (*e*). The antibody also stained the plasma membrane of circulating erythrocytes (*g*). Tissue culture cells were grown on coverslips in minimal essential medium supplemented with 10% calf serum, rinsed in saline, fixed in 100% methanol at -20°C for 4 min, postfixed in acetone at -20°C for 1 min, rehydrated in saline and then processed for immunofluorescence microscopy as previously described¹. Human erythrocytes were diluted in saline, settled on coverslips and fixed with 2% paraformaldehyde before being permeabilized and stained as above. The secondary antibody was tetramethylrhodamine-labelled goat anti-mouse immunoglobulin (USA Biochemicals) at a 1:50 dilution in 20% goat serum. Specimens were examined on a Zeiss photomicroscope III equipped with epifluorescence optics, and fluorescent images were recorded on Plus-X film. Scale bar, 5 μm .



14 to 43 and appear to be randomly intranuclear; they are not associated with the nuclear envelope as judged by through-focus examination at high magnification. The punctate pattern of antibody staining disappears during prophase and reappears in association with the decondensing chromatin during telophase (Fig. 2*b, d*). There is no evidence of fluorescent dots in metaphase or anaphase cells (Fig. 2*c, d*). The spindle fibre staining is apparent even during early prophase when the antibody stains the nascent asters (Fig. 2*b*). No concomitant increase in the staining of the residual interphase microtubule network is observed during this stage. Similarly, the 0-2D5 anti-MAP antibody stains the spindle remnant (midbody) during telophase (Fig. 2*e*), again without significant staining of the arrays of interphase microtubules assembling in the daughter cells. The 0-2D5 antibody does not cross-react with cultured cells of non-human primate, rodent, or marsupial origin, but it does stain the plasma membrane of human erythrocytes (Fig. 2*g, h*). No surface staining has been observed in any other cell type tested.

The origin, molecular weight and cellular distribution of the 0-2D5 MAP distinguish it from the major MAPs of brain microtubule protein. The differences between previously published micrographs⁴ of anti-MAP staining and the punctate nuclear immunofluorescent images shown here suggest that the set of high-molecular weight HeLa MAPs contains structurally and functionally distinct protein species. The 0-2D5 antigen is also distinct from nuclear mitotic apparatus protein (NuMA), another high-molecular weight antigen found in the interphase nucleus and in association with the mitotic spindle of human cells¹¹. The electrophoretic mobility of NuMA and the staining pattern of anti-NuMA monoclonal antibodies (S. E. Winston

and D. E. Pettijohn, personal communication) clearly differ from those of 0-2D5 antigen. NuMA was originally isolated as a non-histone chromatin-associated protein and may be involved in the organization or expression of DNA. At present there is insufficient data to determine whether the foci stained by 0-2D5 have structural or enzymatic activity associated with chromatin or the nuclear matrix.

There are striking similarities between a spectrin-binding protein in human erythrocyte membranes called ankyrin or syndein and the 0-2D5 antigen. Both are easily proteolyzed proteins of molecular weight 200,000 which associate with structural polypeptide polymers (ref. 12; Fig. 1). Antiserum against ankyrin produces spotted nuclear immunofluorescent images and stains the mitotic apparatus in tissue culture cells (V. Bennett, personal communication). Nucleated erythrocytes from birds and amphibians have characteristic circumferential bands of microtubules which contain MAPs¹³; thus the similar distribution of ankyrin and the 0-2D5 MAP raises the intriguing possibility that these antigens have common antecedents as well as similar functional characteristics.

There is no indication, as yet, whether the 0-2D5 antigen, ankyrin or NuMA are necessary spindle components which are sequestered in the nucleus during interphase, or if the antigens function primarily in the interphase nucleus and bind to spindle fibres simply to assure partition into the daughter cells. The rather surprising similarity between an erythrocyte membrane component and a cultured cell MAP suggests that these proteins, or different oligomers of which they are a subunit, may have distinct functions depending on the cellular environment. They may be members of a family of multifunctional molecules like tubulin that are used in different ways according to

the stage of the cell cycle or the programme of cellular differentiation.

The data presented here are consistent with the hypothesis that a subset of mitotic spindle components is sequestered in the nucleus during interphase and that nuclear envelope breakdown initiates or augments spindle assembly by releasing important factors into the cytoplasm^{14,15}. Detergent-lysed cell models do show an increased ability to nucleate aster microtubules after the breakdown of the nuclear envelope¹⁶. Generalizing this theory is difficult in view of the variety of organisms that do not dissolve or fenestrate their nuclear envelopes during mitosis. However, the 0-2D5 antigen appears on early prophase asters,

suggesting that additional mechanisms may be working to allow or promote the association of this antigen with a specific subset of cytoplasmic structures. The preferential association of the 0-2D5 MAP with mitotic apparatus microtubules supports its identification as a spindle MAP and strengthens its candidacy for a mitotic spindle regulatory factor. Microinjection studies are being done to elucidate the role of the 0-2D5 MAP during spindle formation and chromosome separation.

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Rous sarcoma virus-induced phosphorylation of a 50,000-molecular weight cellular protein

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Transformation by Rous sarcoma virus (RSV) is mediated by a single virus-encoded polypeptide, pp60^{src} (refs 1-3), which has protein kinase activity specific for tyrosine residues⁴⁻⁷. The identification of the proteins phosphorylated by pp60^{src} is important in understanding the events involved in RSV-mediated transformation. One potential substrate is a 50,000-molecular weight (MW) phosphotyrosine-containing protein (pp50) which is associated in a complex with pp60^{src} and a cellular 90,000-MW protein (pp90; ref. 8) and thus is immunoprecipitated by antibody to pp60^{src} (refs 6, 9, 10). We have performed further studies to determine whether this 50,000-MW protein (pp50) is present in uninfected cells and whether its phosphorylation at tyrosine depends on RSV-induced transformation. Here we demonstrate that a phosphoserine-containing form of pp50 is present in uninfected chicken cells and that on transformation by RSV, a second form of pp50, detectable by two-dimensional analysis of ³²P-labelled proteins, contains phosphotyrosine as well as phosphoserine. This phosphorylation of pp50 at tyrosine is not temperature sensitive (ts) in cells infected with mutant viruses containing a ts defect in the *src* gene. Previous results⁸ have suggested that the temperature stability of this phosphorylation might be a result of the strong interaction between the mutant pp60^{src} protein and pp50.

To identify pp50 on two-dimensional gels of ³²P-labelled chicken cell lysates, a mixing experiment was performed (Fig. 1). First, using serum from tumour-bearing rabbits (TBR), pp50 was immunoprecipitated from ³²P-labelled chicken cells transformed by the SR-A strain of RSV. Two-dimensional gel electrophoresis of this immunoprecipitate using ampholytes enriched in the pH 5-7 range resolved pp50 (pI = 5.4; Fig. 1a). Comparison of the two-dimensional gel patterns of uninfected cell phosphoproteins (Fig. 1b) with a mixture of the TBR immunoprecipitate and uninfected cell phosphoproteins (Fig. 1c) indicated that the pp50 which is bound to pp60^{src} and precipitated from RSV-transformed cells (pp50A) migrates just to the acidic side of a 50,000-MW phosphoprotein present in uninfected cells (pp50). Although pp50A is not found in unin-

fected cells (Fig. 1b), cells transformed by the SR-A strain of RSV appear to contain both pp50A and pp50B (Fig. 1d). Identical results were obtained using the Prague, B77 and Bryan strains of RSV; transformation-defective deletion mutant virus-infected cell lysates contained pp50B alone. These observations suggest that pp50A might arise by RSV-induced phosphorylation of pp50B. Indeed, charge calibration of these gels by forced carbamylation¹¹ indicated a difference of one charge unit between pp50A and pp50B, which at this pH could be accounted for by a difference of one phosphate group.

To investigate further the possible relationship between pp50A and pp50B, these ³²P-labelled proteins were excised from two-dimensional gels and analysed by partial proteolysis with chymotrypsin, using the method of Cleveland *et al.*¹² (Fig. 2). Several other 50,000-MW phosphoproteins migrating at more basic pH than pp50B were also analysed (Fig. 2, upper panel; pp50C, D and E). pp50B from uninfected cells or cells transformed by the SR-A or Bryan strains of RSV had the same cleavage pattern, consisting of two major phosphopeptides (Fig. 2, lower panel). pp50A from cells transformed by either strain of RSV, and isolated by either two-dimensional gel electrophoresis or immunoprecipitation with TBR serum, yielded two phosphopeptides co-migrating with those of pp50B plus two new phosphopeptides (indicated in Fig. 2 by asterisks). The pp50C, D and E spots do not seem to be related to pp50A and B by this type of analysis, although we have not rigorously ruled out such a relationship. These data suggest that pp50A and B represent different phosphorylated forms of the same protein, pp50A being formed by the transformation-dependent phosphorylation of pp50B at a new site(s).

To determine whether phosphorylation of pp50 on tyrosine⁶ is transformation dependent, the phosphoamino acid contents of pp50A and B were analysed (Fig. 3). The pp50B species from either uninfected (lane 1) or RSV-transformed (lane 2) cells contains only phosphoserine. The transformation-specific pp50A species contains phosphotyrosine in addition to phosphoserine (lane 3). This observation, combined with the phosphopeptide analysis described above, suggests that the difference in migration of the pp50A and B species during isoelectric focusing is due to an additional phosphate group bound to a tyrosine residue on pp50A.

The observation that pp50 is present in both uninfected and RSV-transformed cells, but is phosphorylated on tyrosine only in the latter, suggested that pp50 may be a substrate for pp60^{src}. To test this hypothesis further, we examined whether the tyrosyl phosphorylation of pp50 was temperature sensitive in chicken cells infected with the NY68 strain of RSV¹³, which contains a ts

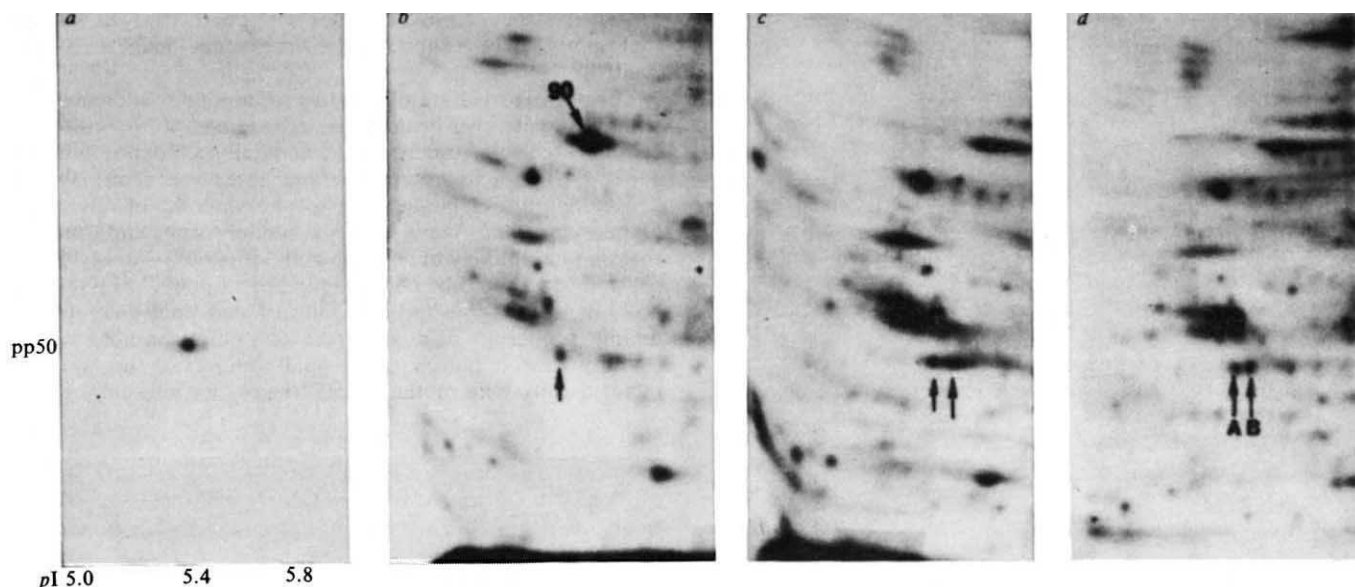


Fig. 1 Two-dimensional analysis of phosphoproteins from normal and RSV-transformed chicken cells. Samples for analysis on two-dimensional gels were prepared as described below. Immunoprecipitated proteins. Chicken cells (1×10^6) transformed by the SR-A strain of RSV were labelled for 4 h with $1 \text{ mCi ml}^{-1} {}^{32}\text{P}$ -orthophosphate in phosphate-free medium. Cell lysates were prepared as described previously¹ using RIPA buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM Tris-HCl pH 7.2) with 5 mM EDTA. The clarified lysates were incubated with 30 μl of TBR serum for 20 min and then formalin-fixed *Staphylococcus* bacteria²¹ were added for 10 min. The washed bacteria were incubated with 30 μl of OFLB (9.5 M urea, 2% NP-40, 5% β -mercaptoethanol, 0.4% ampholytes (LKB) (pH 3.5–10), 1.6% ampholytes (LKB) (pH 5–7) at 22 °C for 10 min. The bacteria were then removed by centrifugation and the supernatant frozen until ready for use. Total cell lysates. Normal or RSV-transformed (SRA strain) cells (1×10^6) were labelled for 4 h with $1 \text{ mCi ml}^{-1} {}^{32}\text{P}$ in phosphate-free medium. The cells were washed three times with STE (150 mM NaCl, 50 mM Tris-HCl pH 7.2, 1 mM EDTA) prepared according to a modification²² of the O'Farrell method²³ for two-dimensional gel analysis of proteins. Briefly, the cells were incubated for 5 min in 300 μl of 2 mM CaCl₂, 20 mM Tris-HCl (pH 8.8) and 50 $\mu\text{g ml}^{-1}$ *Staphylococcus* nuclease buffer (Boehringer-Mannheim). The cells were then forced through a 25 ga. needle, made up to 0.2% SDS, and 0.1 vol of DNase/RNase buffer (1 mg ml⁻¹ DNase I (Sigma), 0.5 $\mu\text{g ml}^{-1}$ RNase A (Sigma), 50 mM MgCl₂ and 0.5 M Tris-HCl pH 7.0) was added. The sample was quick-frozen, lyophilized and immediately resuspended in 180 μl of OFLB. Two-dimensional gels. Samples (15 μl) were loaded on to $0.2 \times 16 \text{ cm}$ cylindrical gels prepared as described elsewhere²³ with a 4:1 ratio of pH 5–7/pH 3.5–10 ampholytes (LKB). After prefocusing, the gels were run at 400 V, for 17 h, followed 800 V for 1.5 h. The gels were then removed from the tube and the gel section from 2–9 cm was equilibrated for 5 min in 2.5% SDS, 5% β -mercaptoethanol, 10% glycerol and 62.5 mM Tris pH 6.8, layered on to a 7.5% Laemmli²⁴ SDS-polyacrylamide gel and electrophoresed until the bromophenol blue reached the bottom of the gel. The gels were dried immediately and exposed to film for 16–24 h using a Lightning-plus (Kodak) intensifying screen. *a*, Phosphoproteins immunoprecipitated with TBR serum. (The pp60^{src} protein does not focus in this pH range and the faint spot around pI 5.6 is pp90). *b*, Phosphoproteins from normal chicken cells. (The spot designated 90 represents the protein which co-migrates with the pp90 immunoprecipitated protein). *c*, Phosphoproteins shown in *b* mixed with the proteins shown in *a*. *d*, Phosphoproteins from RSV-transformed chicken cells.

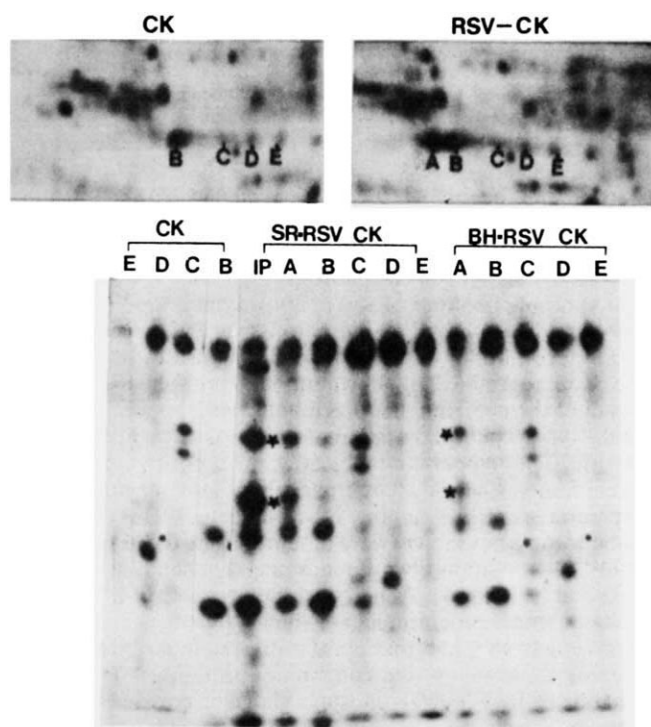


Fig. 2 Partial proteolytic digestion of the 50,000-MW proteins excised from two-dimensional gels. The gels of normal and RSV-transformed (SRA and BH strain) chicken cells were prepared as described in Fig. 1 legend. The upper panel identifies the ${}^{32}\text{P}$ -labelled RSV-transformed chicken cells (RSV-CK) re-electrophoresed (lower panel) on a 12.5% SDS-polyacrylamide gel with 500 $\mu\text{g ml}^{-1}$ chymotrypsin (Sigma) according to the method of Cleveland *et al.*¹². The gel was exposed for 1 week using intensification as described in Fig. 1 legend. The two-dimensional gel pattern of cells transformed by the Bryan (BH) strain of RSV was essentially identical to that of the SR-A strain shown in the upper panels. The lane designated IP contains the pp50 protein immunoprecipitated from ${}^{32}\text{P}$ -labelled RSV (SRA)-transformed cell lysates by TBR serum.

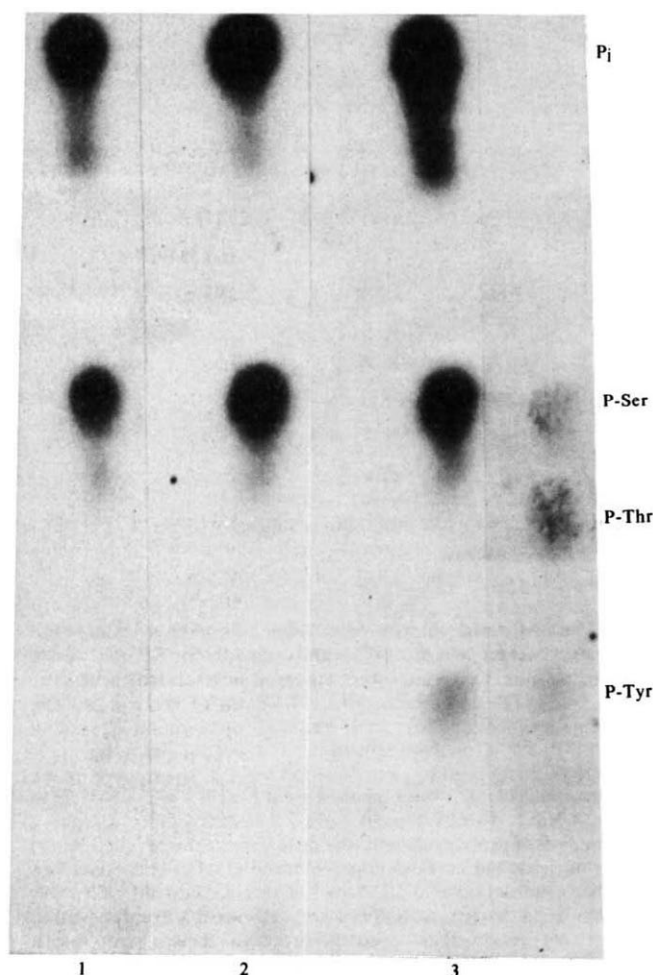


Fig. 3 Analyses of the phosphorylated amino acids associated with the pp50 proteins from normal and RSV-transformed chicken cells. The pp50A and B species were eluted from 12 two-dimensional gels of ^{32}P -labelled proteins from normal and RSV (SR-A) transformed cells prepared as described in Fig. 1 legend. The proteins were TCA-precipitated, acid-hydrolysed and electrophoresed at 4,000 V for 1.75 h in pH 3.5 buffer (pyridine/acetic acid/ H_2O = 1:10:189) as described previously²⁵. Lane 1, pp50B from normal chicken cells; lane 2, pp50B from RSV-transformed chicken cells; lane 3, pp50A from RSV-transformed chicken cells. Phosphoamino acid markers were co-electrophoresed with each radioactive sample. A representative ninhydrin-stained track is shown adjacent to the autoradiogram at the right of the figure for visualization of the marker amino acids (P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine).

defect in the *src* gene. Cells were labelled with ^{32}P -orthophosphate at either 35°C or 41°C and the pp50 bound to pp60^{src} was isolated by immunoprecipitation with TBR serum followed by SDS-gel electrophoresis. Figure 4a shows that the tryptic phosphopeptides of the pp50 protein bound to pp60^{src} cannot be distinguished at the permissive and nonpermissive temperatures. Minor differences in the intensities of the spots were not reproducible. Furthermore, phosphoamino acid analysis of the pp50 immunoprecipitated with pp60^{src} indicated that the tyrosyl phosphorylation of pp50 was not reduced at the nonpermissive temperature (Fig. 4b). As a positive control, it was confirmed that the tyrosyl phosphorylation of pp60^{src} was temperature sensitive in the NY68-infected cells (Fig. 4b). Similar results (not shown) were obtained using the ts *src* mutant LA90¹⁴. In addition, two-dimensional gel analysis of cells labelled at 35°C or 41°C indicated the presence of the pp50A species at both temperatures (not shown).

Thus, we have found that pp50 is present in normal chicken cells as a phosphoserine-containing protein. After transformation by RSV, pp50 is phosphorylated on tyrosine as well as on serine. These results, coupled with the evidence that the

protein is associated in a complex with pp60^{src} (ref. 8), suggest that pp50 may be a substrate for the protein kinase activity of pp60^{src}.

The phosphorylation of previously identified candidate substrates of pp60^{src} has been shown to be temperature sensitive in cells infected with mutant viruses containing a ts defect in the *src* gene¹⁵⁻¹⁷. The pp50 protein described here does not display such a temperature-dependent phosphorylation of tyrosine. Oppermann *et al.*¹⁰ have obtained similar results and conclude that pp50 is unlikely to be a substrate of pp60^{src}. However, our previous data⁸ on the enhanced binding of pp60^{src} to pp50 and pp90 in ts virus-infected cells suggest that pp50 may display unique properties as a substrate of pp60^{src} because of the stability of the pp60^{src}-pp90-pp50 interaction in ts virus-infected cells. The mutant pp60^{src} shows an unusually strong

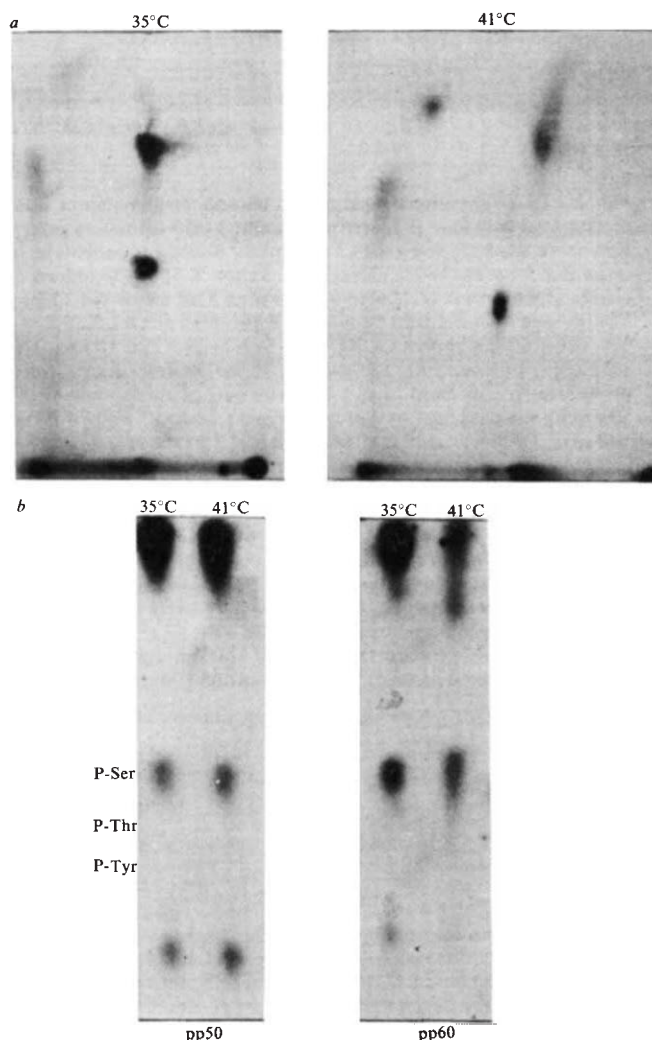


Fig. 4 Phosphopeptide and phosphoamino acid analysis of pp50 associated with pp60^{src} from cells transformed by a ts mutant RSV. Cells transformed by the RSV mutant, NY68 (ref. 13) were incubated at 35°C or 41°C for 24 h and subsequently labelled with ^{32}P (1 mCi ml⁻¹ in phosphate-free medium) for 4 h. Cell lysates were prepared using RIPA and 5 mM EDTA, then clarified and immunoprecipitated with TBR serum as described in Fig. 1 legend. The immunoprecipitated proteins were electrophoresed on a 7.5% SDS-polyacrylamide gel and the pp50 and pp60 proteins eluted by incubation in 0.05 M ammonium bicarbonate and 0.1% SDS for 16 h. The proteins were then precipitated three times with 20% TCA, acetone-washed and split into two fractions. One half of the pp50 sample was oxidized with performic acid, trypsinized and electrophoresed at pH 6.0 as described previously²⁶; the other half was hydrolysed in 6M HCl at 110°C for 2 h, lyophilized and electrophoresed as described in Fig. 3 legend. *a*, Phosphopeptide analysis of pp50 from cells grown at 35°C (left panel) and 41°C (right panel). Chromatography was performed from right to left and electrophoresis, from bottom to top. The origin is in the lower right-hand corner of each autoradiogram. *b*, Phosphoamino acid analysis of pp50 (left panel) and pp60^{src} (right panel) from cells incubated at 35°C and 41°C. Marker phosphoamino acids were co-electrophoresed in each lane and visualized with ninhydrin.

interaction with pp50; up to 90% of mutant pp60^{src} is bound in a complex with pp50 and pp90, while at most 5% of wild-type pp60^{src} is found in this complex⁸. Furthermore, unlike the pp50–pp60^{src}–pp90 complex containing wild-type pp60^{src}, which turns over fairly rapidly, the complex containing mutant pp60^{src} is extremely stable; pulse-chase experiments indicate no detectable dissociation over a 5-h period (our unpublished data). The temperature insensitivity of pp50 phosphorylation might be explained by our previous observation that the binding of ts mutant pp60^{src} to pp50 is very stable at the nonpermissive temperature; thus, it is possible that pp60^{src} is able to phosphorylate pp50 in these conditions, although its ability to phosphorylate other substrates required for transformation is impaired^{15–17}.

The detection of the pp50A species after RSV infection does not seem to result from pp60^{src} binding to an unstable phosphotyrosine-containing form of pp50 present in the uninfected cell as variations in the amount of the pp50–pp60^{src}–pp90 complex in cells transformed by different strains of RSV were not reflected in the levels of the transformation-specific pp50A species. Although the pp50–pp60^{src}–pp90 complex was only minimally detectable in lysates of cells transformed by the Prague strain of RSV⁸, the amount of pp50A species found in these lysates was similar to that found in lysates of cells, transformed by the SR-RSV strain, which contained at least 10-fold greater amounts of pp60^{src} bound to pp50 and pp90. These results suggest that formation of a stable complex between pp50 and pp60^{src} is not required for the detection of the phosphotyrosine-containing form of pp50. It also seems unlikely that pp50 is phosphorylated by a pp60^{src}-activated tyrosine-specific protein kinase as activation of other kinases would be expected to be temperature sensitive in cells infected with ts mutant viruses of RSV. Thus, we conclude tentatively that pp50 is a substrate for pp60^{src}; further testing of this hypothesis, by examining the ability of purified pp60^{src} to phosphorylate pp50 *in vitro*, awaits the purification of the pp50 species containing only phosphoserine (unpublished data).

We have recently obtained evidence that pp50 and pp90 are physically associated with the unrelated transforming proteins of Fujinami sarcoma^{18,19} and Y73²⁰ viruses. Furthermore, we have found that the phosphotyrosine, phosphoserine-containing form of pp50 is detectable on two-dimensional gels of ³²P-labelled lysates from cells transformed by these viruses. This suggests that pp50 and pp90 may have a common interaction with transforming proteins which function as tyrosine-specific protein kinases.

During this study we learned that T. Gilmore, K. Radke and G. S. Martin have also demonstrated the presence of phosphoserine-containing forms of pp50 in uninfected chicken cells.

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Endogenous MuLV infection does not contribute to onset of radiation- or carcinogen-induced murine thymoma

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T-cell lymphoma can be induced in mice by experimental infection with certain strains of murine leukaemia virus (MuLV) or by the action of germ-line transmitted endogenous MuLV¹. B-cell lymphomas are induced in chickens by inoculation with certain strains of exogenous avian leukosis virus². These systems have two common features: a requirement for actively replicating retrovirus, and a long latency period between introduction or appearance of the virus and development of the tumour. Such features are consistent with models of transformation that require multiple rounds of infection either to activate cellular oncogenes by promoter insertion³ or to generate recombinant retrovirus genomes that have novel leukaemogenic activity⁴. The development of T-cell lymphoma in mice exposed to radiation or chemical carcinogens is also marked by a lengthy latency period between exposure to radiation or carcinogen and outgrowth of the tumour, and leukaemogenic MuLV is frequently isolated from tumour tissue^{5,6}. We show here, however, that active infection of cells by endogenous MuLV is not essential to the development of thymic lymphoma in the mouse following exposure to radiation or chemical carcinogens.

The germ lines of all inbred mouse strains contain multiple copies of MuLV sequences, hence the designation endogenous MuLV^{1,7}. A subset of these sequences can be expressed as infectious virus with an ecotropic (mouse-infectious) host range. Relative susceptibility to infection of mouse cells by ecotropic MuLV is regulated by alleles at the cellular *Fv-1* locus⁸. Differing alleles at the *Fv-1* locus, designated *n* and *b*, divide inbred mouse strains into two groups, N-type mice and B-type mice, cells of N-type mice being more susceptible to infection by N-tropic MuLV and those of B-type mice more susceptible to B-tropic MuLV infection. As *Fv-1*-mediated restriction to infection is a dominant phenotype, mice carrying both an *Fv-1ⁿ* and an *Fv-1^b* allele are relatively restrictive for infection by both N-tropic and B-tropic MuLV⁸.

Genetic analysis of the effect of the *Fv-1^b* allele on the incidence of lymphoma in the high incidence AKR inbred mouse strain showed that active infection of somatic cells by endogenous MuLV was required for the development of spontaneous thymic lymphoma⁹. AKR mice carry an *Fv-1ⁿ* allele, and express N-tropic MuLV to high titres. When AKR mice were crossed to mice of the low lymphoma incidence BALB/c strain (homozygous for the *Fv-1^b* allele), both lymphoma incidence and infectious MuLV titre were much lower in F₁ hybrids than in the AKR parent. Suppression of both MuLV expression and spontaneous thymic lymphoma was associated in mice of the (BALB/c × AKR)F₁ × AKR backcross, indicating that the segregating *Fv-1^b* allele coordinately suppressed both traits in a genetically dominant fashion.

A similar experiment described here tested the effect of *Fv-1* on the development of radiation-induced thymic lymphoma, to determine whether this form of induced thymoma also requires active MuLV infection. As shown in Fig. 1a, both the RF/J and BALB/cJ strains are highly susceptible to radiation induction of

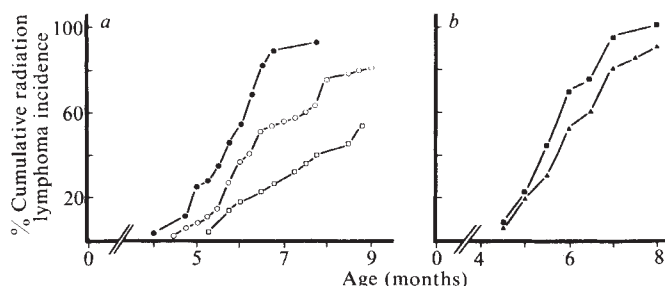


Fig. 1 Cumulative radiation-induced thymic lymphoma incidences in various populations of mice. Female mice were exposed to four weekly doses of 160 rad of γ -ray irradiation⁵ (Gammator M ¹³⁷Cs source, Radiation Machinery Corporation, 530 rad min⁻¹), beginning at 33 $\pm$ 3 days old. Mice were checked daily for signs of lymphoma, including laboured breathing and enlarged peripheral lymph nodes. Mice displaying these symptoms were killed so as to detect the grossly enlarged thymus characteristic of this neoplasia. *a*, Populations of 28 RF/J mice (●—●), 22 BALB/cJ mice (□—□) and 52 (BALB/c $\times$ RF)_{F1} hybrid mice (○—○); *b*, two subpopulations of (BALB/c $\times$ RF)_{F1} $\times$ RF backcross mice, according to *Fv-1* type. 48 *Fv-1*ⁿ homozygous mice (■—■) and 35 *Fv-1*ⁿ/*Fv-1*^b heterozygous mice (▲—▲). *Fv-1* typing was accomplished by typing individual mice for their alleles at the closely linked *Gpd-1* locus¹¹, according to the procedure of Ruddle *et al.*¹².

thymoma, but RF mice display a shorter latency of tumour development. RF mice carry an *Fv-1*ⁿ allele and express N-tropic MuLV, whereas BALB/c mice carry the *Fv-1*^b allele and can express both N-tropic and B-tropic MuLV¹⁰. If infectious spread of these MuLVs promotes development of radiation-induced thymoma in their respective strains, the (BALB/c $\times$ RF)_{F1} hybrid would be expected to show a lower incidence of radiation-induced thymoma than either parental strain, because the RF-derived *Fv-1*ⁿ allele will restrict infection of F₁ cells by the BALB/c B-tropic MuLV, while the BALB/c-derived *Fv-1*^b allele will restrict infection of F₁ cells by the RF N-tropic MuLV. Despite this genetic restriction on virus infection, F₁ hybrids showed a high incidence of radiation-induced thymoma, with a latency intermediate between that of the two parental strains (Fig. 1*a*), thus indicating that restrictive *Fv-1* alleles do not suppress development of radiation-induced thymoma.

To measure the effect of *Fv-1* in this cross more directly, the incidence of radiation-induced thymoma was determined in (BALB/c $\times$ RF)_{F1} $\times$ RF backcross mice typed for the alleles they carried at the *Fv-1* locus. *Fv-1*ⁿ homozygous and *Fv-1*ⁿ/*Fv-1*^b heterozygous backcross mice were identified by typing each backcross mouse for its phenotype at the *Gpd-1*

locus, which is closely linked to the *Fv-1* locus (<1% recombination between the two loci)¹¹. The differing electrophoretic mobilities of the glucose-6-phosphate dehydrogenases specified by the *Gpd-1*^a and *Gpd-1*^b alleles (of RF and BALB/c, respectively)¹² served to distinguish *Gpd-1*^a homozygotes (*Fv-1*ⁿ mice) from *Gpd-1*^a/*Gpd-1*^b heterozygotes (*Fv-1*ⁿ/*Fv-1*^b mice) in the backcross. Figure 1*b* shows that these two subpopulations of the backcross did not differ substantially in their radiation-induced thymoma incidences, confirming that *Fv-1* does not suppress the incidence of this lymphoma.

These findings are not consistent with a requirement for active infection by endogenous MuLV in the development of radiation-induced thymoma. Cellular infection by both ecotropic⁸ and dualtropic MCF-type MuLVs¹³ are sensitive to *Fv-1* restriction, and the leukaemogenic activity of endogenous MuLV is strongly inhibited by a restrictive *Fv-1* genotype⁹ yet here no corresponding strong suppression of radiation lymphoma by *Fv-1* was observed.

When the backcross mice were typed for their haplotype at *H-2*, the major histocompatibility complex of the mouse, no *H-2*-associated effect was observed to influence relative resistance to radiation-induced thymoma. An absence of *Fv-1* and *H-2* influences on thymoma incidence as induced by the chemical carcinogen 3-methylcholanthrene has previously been reported in the I/LnJ $\times$ RF cross¹⁴.

We further assessed the contribution of endogenous mouse-infectious MuLV to the development of radiation- and carcinogen-induced thymoma. If infectious MuLV is indeed aetiologically involved in these induced lymphomas, one would expect to observe association between susceptibility to induced thymoma development and the segregation in appropriate genetic crosses of those murine genes that code for mouse-infectious MuLV. The 129 $\times$ RF cross was chosen for study because RF mice express endogenous ecotropic MuLV¹⁵ but 129 mice have never been observed to do so¹⁶. Although 129 mice contain multiple copies of endogenous MuLV genes⁷, and indeed express virus-coded antigens *in vivo*¹⁷, their DNA lacks a specific portion of the endogenous ecotropic MuLV genome¹⁶ and hence complete infectious ecotropic MuLV is not elaborated in 129 tissue. Furthermore, we have observed that RF mice contain a single locus governing iododeoxyuridine (IUDR)-induction of XC-detectable ecotropic MuLV. Such loci are thought to represent structural genomes of endogenous MuLV¹⁸. The conclusion that RF mice carry a single replication-competent ecotropic MuLV genome was reached by *in vitro* IUDR treatment of cultures of tail explant fibroblasts obtained from individual mice of the (129 $\times$ RF)_{F1} $\times$ 129 backcross, followed by detection of MuLV production by these cells using

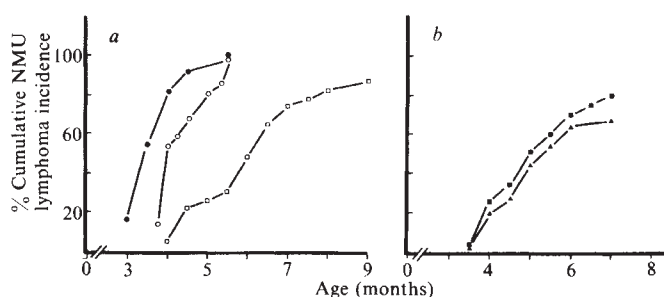


Fig. 2 Cumulative nitrosomethylurea-induced thymic lymphoma incidences in various populations of mice. Female mice were injected weekly with NMU (2 mg ml⁻¹ in SSC, 30 mg per kg body weight) intraperitoneally for 5 consecutive weeks, beginning at 5 weeks of age²³. Development of lymphoma was monitored as described in Fig. 1 legend. *a*, Populations of 39 RF/J mice (●—●), 23 129/J mice (□—□), and 23 (129 $\times$ RF)_{F1} hybrid mice (○—○); *b*, two subpopulations of the (129 $\times$ RF)_{F1} $\times$ 129 backcross, 71 mice containing an IUDR-inducible, XC-detectable ecotropic MuLV genome (■—■), and 61 mice lacking this MuLV genome (▲—▲).

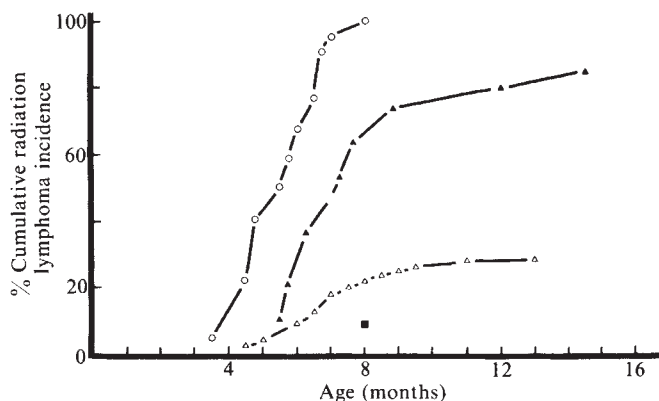


Fig. 3 Cumulative radiation-induced thymic lymphoma incidences in various populations of mice. Female mice were exposed to γ -ray irradiation and monitored for development of thymic lymphoma as described in Fig. 1 legend. 22 RF/J mice (○—○), 11 129/J mice (■—■), 19 (129 $\times$ RF)_{F1} hybrid mice (▲—▲) and 197 (129 $\times$ RF)_{F1} $\times$ 129 backcross mice (△—△). Only one of the eleven 129/J mice developed thymoma, and thus this result is presented as a single point.

the XC assay¹⁹. This procedure has been described by Lander *et al.*²⁰, and detects the presence of ecotropic MuLV in all RF and (129×RF)_F₁ mice, but not in 129 mice. In a population of (129×RF)_F₁ × 129 backcross mice, tail cultures from 72 of 132 mice tested were virus-positive, demonstrating the segregation of a single RF-derived ecotropic MuLV locus in the cross. Note that this assay says nothing about the manner or quantity of expression *in vivo* of ecotropic MuLV in the mice tested. It merely detects the presence of a replication-competent ecotropic MuLV genome while allowing individual mice to be tested concomitantly for radiation and carcinogen-induced thymoma development. In contrast to the BALB/c × RF cross described above, RF and 129 mice do not differ with respect to their *Fv-1* genotype⁸, and RF ecotropic MuLV expression will not be restricted in this cross as compared with the parental RF strain.

Figure 2a shows that both RF and 129 mice are highly sensitive to induction of thymoma by the chemical carcinogen *N*-nitrosomethylurea (NMU), and that RF mice display a shorter latency of tumour development than do 129. *F*₁ hybrid mice have an intermediate latency. The high incidence of NMU-induced thymic lymphoma in the 129 strain, albeit at later ages than RF, in itself demonstrates that cellular infection by ecotropic MuLV is not required for the development of this induced lymphoma, because, as mentioned above, 129 mice lack this viral activity. When the incidence of NMU-induced thymoma was compared in (129×RF)_F₁ × 129 backcross mice either carrying or lacking a replication-competent ecotropic MuLV genome, as typed by IUdR induction of tail explant fibroblast cultures followed by detection of MuLV expression using the XC plaque assay²⁰, the two subpopulations of the backcross showed virtually identical incidences (Fig. 2b). This indicates that the RF ecotropic MuLV locus detected here does not seem to be a major determinant in the short latency of NMU-induced thymic lymphoma in RF mice.

Figure 3 shows that RF mice are much more sensitive to radiation-induced thymoma than are 129 mice, and that *F*₁ hybrid mice also develop a high incidence of this tumour. A population of (129×RF)_F₁ × 129 backcross mice was exposed to radiation (Fig. 3), and backcross mice that developed thymic lymphoma were randomly tested for the presence of the RF IUdR-inducible ecotropic MuLV locus. Only 22 out of 37 lymphomatous backcross mice tested were seen to carry the RF ecotropic MuLV locus, a result not significantly different from that expected from random segregation of this locus in the lymphomatous population, and indicating that the locus is not a major factor in determining susceptibility to radiation-induced lymphoma.

These findings point to a basic difference between the aetiologies of spontaneous and carcinogen- or radiation-induced thymoma. The spontaneous occurrence of this tumour is associated with expression of endogenous mouse-infectious MuLV activities, including both XC-positive⁹ and XC-negative²¹ ecotropic MuLVs and dualtropic MCF MuLV¹³. The present data indicate that both carcinogen- and radiation-induced thymoma develop in the absence of replication-competent XC-positive ecotropic MuLV activity. The dependence of spontaneous thymoma development on actual infectious events has been previously indicated by its marked susceptibility to suppression by a restrictive *Fv-1* allele⁹. In contrast, neither carcinogen- nor radiation-induced thymoma development is sensitive to suppression by *Fv-1*. This indicates that those infectious viral events that are aetiologically involved in development of spontaneous thymomas are not required for the development of induced thymomas. Finally, note that the possible importance to the development of induced thymomas of endogenous MuLV antigen expression in the absence of infectious virion production²² has not been addressed here, and remains a viable hypothesis.

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Altered methylation of endogenous viral promoter sequences during mammary carcinogenesis

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Mouse mammary tumour virus (MMTV) is a type B retrovirus that may be transmitted either by somatic cell infection via infectious virus borne in mother's milk or vertically by passage of virus-specific sequences in the germ line. Endogenous viral sequences act as stable genetic loci¹ and are found in all inbred mouse strains studied. Available data suggest that, in general, strains exhibiting only endogenous virus have a low tumour incidence whereas a high tumour incidence occurs in strains carrying milk-borne MMTV, in addition to germ-line sequences. However, as removal of the milk-borne virus does not completely eliminate tumours, endogenous proviral sequences may also be involved in tumorigenesis^{2–4}. Recent results have shown that proviral sequences acquired from milk are undermethylated (low 5-methylcytosine content), whereas endogenous MMTV sequences are highly methylated. An inverse relationship between proviral methylation and transcriptional activity has also been demonstrated^{5,6}. This model suggests that an important mechanism of MMTV-induced tumorigenesis in uninfected mice (those lacking milk-borne MMTV) may involve derepression of endogenous proviruses, possibly through demethylation of these sequences. Here we show that the methylation pattern of endogenous virus sequences is specifically altered during tumorigenesis in uninfected mice.

Several tumours occurring in BALB/c mice were studied both for the acquisition of new MMTV proviral sequences and for demethylation of endogenous viral sequences. As tumours occur infrequently in this strain, those derived from the D1 series of hyperplastic outgrowth lines developed by Medina⁷ were used. These tissues (supplied by R. Cardiff) are transplantable and are analogous to the pre-neoplastic nodules commonly seen in the mammary gland. Outgrowth lines are

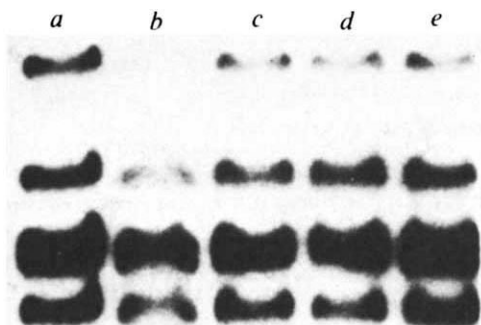


Fig. 1 *EcoRI* digestion of DNA for liver and mammary tumours of the BALB/c mouse strain. DNA was extracted by placing tissue in DNA extraction buffer (0.2 M Tris-HCl pH 8.1, 0.01 M EDTA pH 7.0, and 0.1 M NaCl) and homogenized using a motor driven, Teflon-glass Dounce homogenizer. Protein was removed by incubation at 37 °C for 12 h with proteinase K (100 g ml⁻¹) and SDS (1%) followed by phenol-chloroform (2:1) extraction. DNA was spooled on to a glass rod by addition of 0.2 M sodium acetate and two volumes of cold ethanol. DNA was resuspended in 0.005 M Tris-HCl pH 7.4 and 0.1 mM EDTA pH 7.0 and concentration determined by UV absorption. Each sample (10 µg) was digested to completion by *EcoRI* (12-fold excess) as determined by using λ bacteriophage DNA as an external control. Samples were electrophoresed in 0.8% agarose in a buffer containing 0.02 M sodium acetate pH 7.0, 0.018 M NaCl and 0.002 M EDTA. Bromophenol blue was used as a tracking dye which was electrophoresed 20 cm. ³²P-labelled, *HindIII*-digested λ bacteriophage DNA was run in parallel as a molecular weight marker. Following electrophoresis DNA was transferred to nitrocellulose sheets (Schleicher & Schuell) by the method of Southern¹¹ using modifications as described previously⁵. Virus-specific bands were detected by hybridization with ³²P-labelled, representative, complementary DNA (cDNArep) synthesized using calf thymus primers to initiate DNA synthesis by reverse transcriptase on a cloned MMTV DNA template. The hybridization buffer included 50% formamide, Denhardt's buffer¹¹, 20 g ml⁻¹ salmon sperm DNA, 0.05 M HEPES buffer pH 7.0, and 3×SSC (SSC=0.15 M NaCl, 0.015 M Na citrate). After hybridization at 41 °C for 48 h, filters were washed as described previously⁵ and bands detected by autoradiography using Kodak XAR-5 X-ray film with Cronex Lightning Plus intensifying screens at -70 °C. *EcoRI* was used to digest DNA from BALB/c liver (lane a), D1 mammary tumours 997, 968 and 599 (lanes b-d, respectively) and the transplanted mammary tumour 1247 (lane e). Molecular weight in kilobases was determined by inclusion of *HindIII*-digested λ bacteriophage DNA (not shown).

transplanted into young females along with pituitary isografts to facilitate tumour development.

Virus-specific sequences were detected using the method of Southern⁶ following digestion of DNA with *EcoRI*, which has a single restriction site within the full-length MMTV genome. Therefore, cleavage by this enzyme generates from each provirus two virus-specific fragments whose sizes depend on the integration site of the virus in the host genome (thus, fragment length is determined by the nearest *EcoRI* site in cellular DNA). Using this technique we determined that BALB/c mice contain three endogenous MMTV proviruses, designated units I, II and III; units II and III are full length and unit I is subgenomic.

Figure 1a is an *EcoRI* digest of uninfected liver⁹ DNA, showing the five previously mapped endogenous virus-specific fragments which range in size from 6.7 to 16.7 kilobases (kb). Unit I is included in the 16.7-kb fragment, unit II in fragments of 8.5 and 7.0 kb and unit III in the 10.0- and 7.8-kb fragments. Examination of DNA from BALB/c tumours (Fig. 1b-e) reveals virus-specific fragments identical to those found in liver DNA. Because mammary tumours are clonal¹⁰ and acquisition of MMTV proviruses is known to be a random event in the mouse genome¹, the failure to find additional *EcoRI* fragments whose size is dependent on site of integration (see above and ref. 11) indicates that only the genetically transmitted viral sequences are present. Therefore, infection and re-integration of MMTV DNA, although facilitating mammary carcinogenesis, is not essential.

The level of 5-methylcytosine in virus-specific sequences was probed using the restriction enzymes *MspI* and *HpaII*, isoschizomers which recognize the sequence 5'CCGG. Because *MspI* will cleave this sequence regardless of methylation of the internal cytosine residue, while *HpaII* is inhibited by methylation at this site^{5,12}, digestion of cellular DNA with these enzymes differentiates methylated from non-methylated sites. Figure 2 shows *MspI* and *HpaII* digestion of BALB/c liver and D1 tumour DNAs. *MspI* digestion of liver DNA (Fig. 2a) generates a series of virus-specific fragments of 0.2-8.4 kb (ref. 5). *HpaII*-cleaved BALB/c liver DNA, on the other hand, demonstrates extensive methylation of all viral fragments, as evidenced by the single high molecular weight (>15 kb) virus-specific fragment. *HpaII* digestion of D1 tumour DNAs (Fig. 2d, f, h), however, generates two virus-specific bands of 2.1 and 1.4 kb. Therefore, sites in MMTV proviruses methylated in DNA from both uninfected liver and lactating mammary gland (Fig. 2b, and ref. 5) are demethylated during mammary carcinogenesis in the BALB/c mouse strain.

We determined the origin of the 2.1- and 1.4-kb *HpaII* fragments by comparing *MspI* digests from various inbred mouse strains, as MMTV proviral units vary between different strains¹. Comparison of the previously published⁵ *MspI* digestion of DNA from the CBA/CaJ mouse strain (which includes units II and III) with that obtained with DNA from BALB/c (which includes units I, II and III), indicated that the 2.1- and 1.4-kb *MspI*-*HpaII* fragments are derived from the subgenomic unit I.

HpaII digestion of DNA from BALB/c mammary tumour 1247 (not shown) produced several virus-specific fragments in addition to those derived from unit I, indicating that demethylation of other endogenous proviruses has occurred in this tumour. The origin of these additional sites of demethylation in tumour 1247 was identified by *EcoRI*-*HpaII* double digestions. By determining which *EcoRI* bands are sensitive to further cleavage by *HpaII*, one can ascertain the proviral units being demethylated during tumorigenesis (Fig. 3). This is possible in the BALB/c strain because all *EcoRI* fragments are mapped to specific units of MMTV proviral DNA⁹.

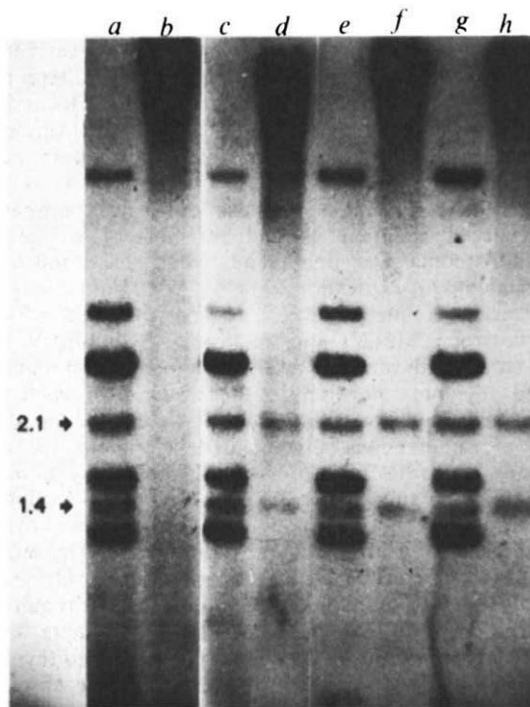


Fig. 2 Methylation of MMTV sequences in BALB/c liver and mammary tumours. DNA from either liver (lanes a, b) or D1 mammary tumours (lanes c-h) were digested with either the methyl-insensitive isoschizomer *MspI* (lanes a, c, e, g) or methyl-sensitive *HpaII* (lanes b, d, f, h) and virus-specific bands analysed as described in Fig. 1. legend.



Fig. 3 Characterization of demethylated sequences in mammary tumour 1247. DNA from tumour 1247 was digested with either *EcoRI* (lane *a*) or sequentially with *EcoRI* and either *MspI* (lane *b*) or *HpaII* (lane *c*) and virus-specific fragments were detected as described in Fig. 1. legend.

Cleavage of MMTV-specific *EcoRI* fragments by the methyl-insensitive isoschizomer *MspI* (Fig. 3*b*) indicates the presence of *MspI*-*HpaII* sites in all fragments. Double digestion with *EcoRI* and *HpaII* (Fig. 3*c*) reveals demethylation of specific proviral sequences. The 8.5- and 7.8-kb fragments remain methylated as evidenced by their presence in the *EcoRI*-*HpaII* double digestion. The 16.7, 10.0- and 6.7-kb *EcoRI* fragments, however, are demethylated because, whereas *MspI*-*HpaII* sites in BALB/c liver are highly methylated, resulting in no cleavage of any *EcoRI* fragments by *HpaII* (data not shown), tumour 1247 showed a specific loss of methylated bases from these three fragments. Previous data⁹ mapping restriction endonuclease sites on the endogenous MMTV proviruses of BALB/c mice revealed that the 8.5- and 7.8-kb *EcoRI* fragments are derived from the 5' ends of units III and II, respectively. Two of the remaining three *EcoRI* fragments (10.0 and 6.7 kb) which are cleaved by the methyl-sensitive *HpaII* are derived from the 3' ends of units II and III. The 16.7-kb *EcoRI* fragment is obtained from the subgenomic, 3' sequence containing unit I. Thus, spontaneous BALB/c tumours studied show the specific demethylation of virus-specific sequences derived from the 3' region of the viral genome. Recent sequencing data¹³ indicate the presence of *HpaII* cleavage sites in the long terminal repeats (LTRs) of several MMTV proviruses which seem to be identical to units II and III. The demethylation event occurring in tumour 1247 is thus extremely specific, as it distinguishes between two direct repeats occurring at each end of these two proviruses⁹. The significance of these additional demethylation events in tumorigenesis of this particular tumour is unclear, because in other tumours demethylation of only unit I was observed.

Previous studies¹⁴⁻¹⁶ have indicated that, at each end of retroviral DNA, sequences from the 3' end of the viral genome are repeated along with a short sequence from the 5' region. In MMTV this LTR is 1,200 base pairs long and contains an open reading frame capable of encoding¹³ a protein of molecular

weight 38,000. As hypomethylation correlates with gene expression, the demethylation observed in these spontaneous BALB/c tumours may represent activation of this gene. However, no protein derived from this open reading frame has been identified.

Another possible explanation for the specific demethylation of these sequences during mammary carcinogenesis involves promoter activation. The promoter for transcription of the retroviral genes apparently resides in the 3' sequences of the LTR^{17,18}, and it has been suggested that carcinogenesis occurs through a mechanism of cell transformation referred to as oncogenesis by promoter insertion, that is, that the introduction of the viral promoter (in the LTR) allows transcription of cellular sequences associated with the 3' end of the provirus. The present results suggest that in addition to transformation by promoter insertion that may occur during exogenous MMTV infection, carcinogenesis by promoter derepression may occur. This is supported by the observed demethylation of either the defective MMTV sequence (unit I) or specifically the 3' half of the nondefective proviruses (units II and III), as decreased levels of methylation are known to correlate with increased transcriptional activity of MMTV^{5,6} and other eukaryotic genes^{19,20}.

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High frequency of gene transfer after fusion between bacteria and eukaryotic cells

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Recent studies¹ have demonstrated that eukaryotic simian virus 40 (SV40) genes carried in a bacterial plasmid can be directly transferred into mammalian cells in culture; after polyethylene glycol (PEG)-induced fusion with bacterial protoplasts, a fraction of the recipient cells yielded infectious virus. We have now investigated whether this procedure can also produce stable transformants. We report here that the efficiency of focus formation after transfer of either polyoma virus or SV40 early genes was at least equal to that observed after infection with virions. At high ratios of bacteria/recipient cells, all the cells were observed to express the early viral proteins 48 h after fusion. In optimal conditions, transfer by fusion thus seems to be 10- to 20-fold more efficient than DNA transfection by the Ca²⁺ co-precipitation technique² for the introduction of foreign genes into eukaryotic recipients.

Escherichia coli (strain 1106; ref. 3) carrying one of the plasmids listed in Table 1 were grown to a density of 2×10^8 cells per ml. Chloramphenicol was added at a final concentration of $200 \mu\text{g ml}^{-1}$ and the cultures were aerated overnight. Aliquots (10 ml) were centrifuged and the bacteria resuspended at 0°C in $100 \mu\text{l}$ of 50 mM Tris-HCl buffer, pH 8.0, containing 20% sucrose; $20 \mu\text{l}$ of a freshly made solution of lysozyme (10 mg ml^{-1} ; Boehringer) were added and the suspension incubated at 0°C for 10 min. After addition of $50 \mu\text{l}$ of 0.25 M EDTA, the suspension was diluted by progressive addition of 1.3 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 9% sucrose. Microscopic examination indicated that $>90\%$ of the bacteria were converted into protoplasts. The recipient cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) in 35-mm Petri dishes to a density of 2×10^5 cells per plate. The medium was removed and the cells were washed once with 2 ml of Tris-buffered saline solution, 2 ml of the protoplast suspension were added to the plates and centrifuged onto the cell layer (1,500 r.p.m. for 3 min). The supernatant was removed and 0.5 ml of a 48% solution of PEG 1,000 in DMEM (48 g PEG dissolved in 52 g DMEM) was added. After 1 min at room temperature, the cells were washed carefully five times with Tris-buffered saline before being returned to the growth medium (DMEM supplemented with 10% newborn calf serum (Gibco)). The medium was changed on alternate days and the cultures were checked after 10 days for the appearance of foci.

As shown in Fig. 1, transformed foci were observed after fusion of FR3T3 rat cells⁴ with bacteria carrying plasmid pPY-1. This plasmid includes a full-size wild-type polyoma virus genome recombined with pBR322 at the *Bam*HI site in the late viral region. No foci were produced after fusion with bacteria carrying an intact pBR322 plasmid, and results obtained with recombinants carrying various polyoma virus mutants were always in agreement with the known transforming ability of the viral genes present in the plasmid (Table 1): cleavage of polyoma virus DNA by recombination at the *Eco*RI site in the early region did not interfere with its transforming ability (plasmid pTS25E), as previously demonstrated after DNA transfection⁵⁻⁷, whereas no transformants were produced using the cloned genome (pNG-1) of a transformation-defective *hrt* mutant⁸. The efficiency of focus induction was ~ 40 transformants per 2×10^5 cells at an input ratio of 10^4 protoplasts per cell (Fig. 1). Identical efficiencies were observed (data not shown) after fusion of protoplasts carrying either polyoma or SV40 early genes in pBR322 plasmid (plasmids pPY-1 and pSV-1; see Table 1) with both mouse and rat embryo fibroblasts in primary cultures, suggesting an equally high frequency of gene transfer. These values are comparable with those observed after infection of the same cells with high multiplicities of viral particles⁴.

As 90–100% of the cells expressed the early proteins (T antigens) after virion infection, the relatively high efficiency of

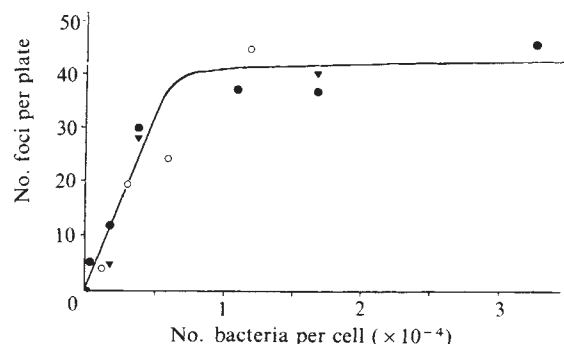


Fig. 1 Efficiency of focus formation after fusion of FR3T3 cells with bacteria carrying genomic polyoma virus DNA inserted in pBR322. Focus formation was measured as described in ref. 4. Abscissa, ratio of number of bacterial protoplasts to number of cells. Ordinate, number of foci per 35-mm Petri dish (2×10^5 cells). The different symbols represent results obtained in three separate experiments.

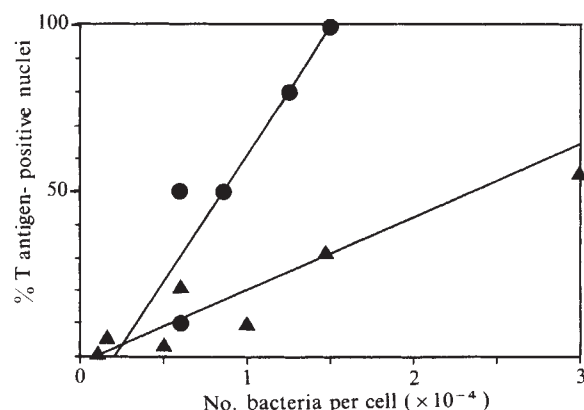


Fig. 2 Expression of the early viral antigens after fusion of FR3T3 (Δ) and CV1 ($\bullet$) cells with bacteria carrying SV40 early genes inserted in pBR322.

focus formation after fusion suggested a comparable high frequency of gene transfer. This frequency was measured directly by immunofluorescence determination on fixed cells after transfer of the early region of SV40 (plasmid pSV-1; see Table 1) into either permissive CV1 cells or non-permissive FR3T3 cells. Results are shown in Fig. 2. As many as 100% of CV1 nuclei were stained at a bacteria/recipient cell ratio of $\sim 10^4$. The dose-response curve exhibited a different slope in the case of FR3T3 rat cells, the efficiency of transfer being about one-half that observed for CV1.

Further studies are needed, as such differences between cell lines may reflect different efficiencies of the fusion process; they may reflect different efficiencies of expression after transfer of a given gene (for example, FR3T3 cells are non-permissive whereas CV1 cells are fully permissive for the development of SV40 virus). Frequencies of T-positive cells $>50\%$ were observed in FR3T3 cells at an input ratio of 3×10^4 bacteria per cell. It may be technically difficult to increase this ratio further, because a significant fraction of the cells are killed at very high bacteria/cell ratios.

The transfer frequencies observed were at least one order of magnitude higher than those after DNA transfection, which is

Table 1 Efficiency of focus formation after fusion with recipient cells of protoplasts carrying various viral DNA fragments in different plasmids

Plasmid	Viral DNA fragment	Insertion site in pBR322	Focus formation (foci per 2×10^5 cells)*
pBR322	None	—	0
pPY-1	Genomic polyoma DNA (wild-type A2 strain) cleaved at the <i>Bam</i> HI site	<i>Bam</i> HI	40
pTS25E	Genomic <i>tsa25E</i> polyoma DNA cleaved at the <i>Eco</i> RI site	<i>Eco</i> RI	25
pNG-1	Genomic polyoma <i>hrt</i> NG 18 mutant cleaved at the <i>Bam</i> HI site	<i>Bam</i> HI	0
pMC-2	Wild-type polyoma A2 sequences from <i>Bam</i> HI to <i>Eco</i> RI sites (distal parts of early and late genes)	<i>Eco</i> RI, <i>Bam</i> HI	0
pSV-1	Wild-type SV40 sequences from <i>Bam</i> HI to <i>Hpa</i> II sites (complete early region)	<i>Eco</i> RI	32

Plasmids pPY-1, pTS25E, pNG-1 and pMC-2 were constructed in our laboratory by the usual procedures (P. Clertant and M. Canning, unpublished results); plasmid pSV-1 is described in ref. 10 and was obtained from Dr C. Benoist. A2 strain polyoma DNA, see ref. 11; mutant *tsa25E*, ref. 12; mutant *hrt* NG 18, ref. 8.

* FR3T3 cells were fused with bacterial protoplasts as described in the text.

restricted to only a few 'competent' cells⁹. In our experiments, transfer of either polyoma or SV40 virus early genes by the Ca^{2+} co-precipitation technique produced 5–10% T antigen-positive cells at high doses of DNA (10 μg per 10^5 cells).

The frequency of T-positive CV1 cells after fusion was also 15- to 20-fold higher than that reported in comparable experiments¹. A systematic analysis of the parameters involved (data not shown) indicated that the extent of lysozyme treatment was critical. As expected, incomplete conversion into protoplasts led to reduced frequencies of transformation. The same result was also observed, however, for prolonged lysozyme attacks which led to the lysis of a fraction of the protoplasts, apparent from the increased viscosity of the suspension. On the other hand, the chloramphenicol amplification step seems to be essential: in several cases where this step was omitted, using either pBR322 or pAT153 plasmid, no transformants were produced after

fusion (data not shown). However, as these points were checked in previous experiments (W. Schaffner, personal communication), another important parameter that must be considered is the choice of bacterial strain. Although we cannot provide comparative data, it is possible that the strain used here (*E. coli* 1106) is more efficient than others, for example, *E. coli* HB101 (ref. 1), in producing stable protoplasts.

Biohazards associated with the experiments described here have been examined previously by the French National Committee and the experiments were carried out according to the rules established by this Committee.

We thank L. Carbone, F. Tillier and M. L. Varani for technical assistance. This work was supported by grants from the CNRS (ATP "Biologie Moléculaire du Gène") and the Institut National de la Santé et de la Recherche Médicale (AT 79-104, PRC "Génie Génétique").

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Circular double-stranded RNA in potato spindle tuber viroid-infected tomatoes

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Viroids are circular molecules of single-stranded RNA consisting of ~360 nucleotides^{1,2}, and the mechanism by which these smallest of all known infectious agents replicate in host cells has been extensively studied. Earlier work suggested that viroids replicate using DNA intermediates³, but rigorous hybridization experiments^{4,5} failed to detect DNA complementary to potato spindle tuber viroid (PSTV) in infected plants, and there is no specific inhibition of viroid synthesis⁶ by actinomycin D. There is strong evidence that the replication of viroids involves RNA intermediates. *Citrus exocortis* viroid (CEV)^{7,8}-infected and PSTV-infected^{4,9,10} tissues have been shown to contain RNA that is complementary to the infecting viroid, and recently it was shown that DNA-dependent RNA polymerase II, isolated from tomato, will produce full-length complementary copies of purified viroids *in vitro*¹¹. Because much of the RNA complementary to CEV was shown to be double stranded, it could be anticipated that viroid-infected tissue contains unique species of double-stranded RNA (dsRNA). Using serologically specific electron microscopy¹², we have now detected molecules of circular dsRNA in crude extracts of PSTV-infected tomato. These molecules have contour lengths that would be expected for a double-stranded intermediate of PSTV.

Using antiserum to dsRNA, serologically specific electron microscopy was used to demonstrate the involvement of dsRNA in the replication of tobacco mosaic virus¹³. Similar assays of PSTV-infected tomato tissue revealed several linear and relaxed circular molecules of dsRNA, whereas extracts of healthy tomato tissue contained a similar number of linear molecules of dsRNA but approximately 10 times fewer circular molecules.

Tomato (*Lycopersicon esculentum* cv. Rutgers) leaves were powdered with liquid nitrogen and ground with 0.1 M Tris-HCl pH 8.0, containing 10 mM Na_2EDTA (Tris) (1 ml per g of tissue). The following steps were done at room temperature. Electron microscope grids with carbon-reinforced Formvar films were floated on antiserum to dsRNA, diluted 1/500 with Tris, for 30 min. After washing with Tris the grids were placed

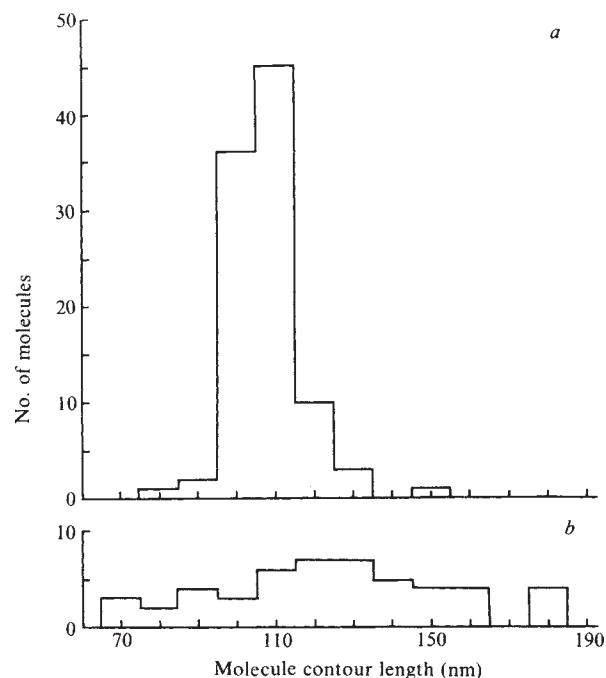


Fig. 1 Histograms of the circumference of circular dsRNA found in extracts of PSTV-infected (a) and healthy (b) tomato tissue. Electron micrograph negatives of grids prepared as described in the text were projected and the circular forms were traced and measured. We measured a total of 50 molecules from 18 negatives of healthy and 100 molecules from 3 negatives of PSTV-infected tissue extracts.

on drops of extract for 2.5 h. The grids were washed with Tris and distilled water, dipped in 95% ethanol for 30 s, rinsed with distilled water and placed on drops of 0.1 mg ml^{-1} cytochrome c in Tris for 15 min. The grids were washed with distilled water and placed on drops of 0.01 M phosphate pH 7.2 for 15 min, then removed from the phosphate and held for 30 s in a stream of drops of 1% aqueous uranyl acetate from a motor-driven syringe equipped with a 0.2- μm filter. They were rinsed by touching to a drop of distilled water for 1 s and blotted dry, then examined under the electron microscope.

The assay was specific for dsRNA: neither circular nor linear forms were seen when normal serum or antiserum to a DNA-RNA hybrid were used in the assays. Moreover, the anti-dsRNA serum used (provided by R. M. Lister) does not react

with DNA or single-stranded RNA¹⁴. Assays of PSTV preparations purified by polyacrylamide electrophoresis¹⁵ were negative, confirming an earlier report that PSTV does not react with antiserum to dsRNA¹⁶.

The length of denatured PSTV has been shown to be 100 nm (ref. 17) and 110 nm (ref. 18). Using a linear density of 2.4×10^6 daltons μm^{-1} for dsRNA and a mass of 250,000 daltons, the calculated length of a dsRNA intermediate of PSTV is 104 nm. These lengths compare well with the modal circumference of 110 nm found for the circular dsRNA from PSTV-infected

tissue (Fig. 1a). The random distribution of sizes of the circular molecules of dsRNA found in healthy tissue suggests they may be closures of linear dsRNA (Fig. 1b).

With spherical viruses, the detection limits of serologically specific electron microscopy have been shown to be 10^9 virions per ml (ref. 19). As the number of circular forms attaching to grids decreases rapidly on dilution, the concentration of circular dsRNA in viroid-infected tissue must be of similar magnitude, which is consistent with the low rate of viroid accumulation¹⁵ in the plant.

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A + T-rich linkers define functional domains in eukaryotic DNA

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Although an increasing number of detailed DNA sequence studies are being carried out, little is known about the long-range organization of the eukaryotic genome in relation to chromosomal organization and to functional units of transcription, replication, meiotic crossing-over and nuclear architecture. Based on electron microscope (EM) observations of partially denatured DNA of duck, mouse and rat, we have recently reported a systematic punctuation of DNA by early melting zones ('A + T-rich linkers')¹ ~800 base pairs (bp) long which are either clustered or at intervals of 10–40 kbp. In an attempt to correlate this feature with the organization of specific genes, we examined cloned genomic DNA from chick and *Drosophila*, including the globin gene family, and an isolated high-molecular-weight mRNA. We show here that the chick α -globin gene cluster is framed by a cluster of A + T-rich linkers at the 5' end and by an isolated linker at the 3' end which maps close to the sites of transcription termination and DNase hypersensitivity. The resulting frame of ~15 kbp correlates well with the maximum size of globin gene transcripts found at the pre-mRNA level. The β -globin gene cluster is also framed, 5' by a single A + T-rich linker and 3' a cluster of four linkers. In *Drosophila*, the single mRNA sequence of ~3.3 kbp for the ecdysone-inducible P1 protein is similarly delimited by A + T-rich linkers. A + T-rich linkers thus seem to define domains of the eukaryotic genome which may correlate with putative functional units. The specific organization observed in total DNA of duck, mouse, rat, man, chicken and *Drosophila* is confirmed in gene-specific cloned genomic DNA.

Cloned genomic recombinants including the α - and β -globin gene clusters were taken from the chicken 'library' of Dodgson *et al.*². The globin genomic maps shown in Fig. 1 are based on recent publications^{3–5} and unpublished data (J. Dodgson, personal communication). Justification of the experimental approach and general methods of partial DNA denaturation and spreading for EM have been reported previously¹. Briefly, individual recombinant DNA cloned in λ Charon 4A, was denatured at a concentration of 0.5–0.8 $\mu\text{g ml}^{-1}$ by 85% formamide (24.5°C), spread by the Kleinschmitt technique, taken up on collodion-coated grids, shadow-casted with

palladium/platinum and observed in a Siemens Elmiskop 101 at a primary magnification of $\times 10,000$. The length of the DNA molecules and the positions of melted regions were measured and statistically analysed by a digitizer branched on a HP 9830 calculator. Figure 2 shows two examples of the original micrographs of such partially denatured DNA molecules.

To determine the 5'–3' orientation with respect to the mRNA sequences and to begin compilation of denatured regions, we used the characteristic denatured regions in the short arm of the λ vector DNA serving also as a very precise 'internal thermometer'. Out of hundreds of molecules observed, a random sample of 20–30 full-length recombinants was measured for any one clone. The data were then realigned with one of the denaturations observed in the chicken DNA inserts so that the position of the melted regions could be plotted precisely. The accuracy of the measurements was of the order of 0.03 nm or ~100 bp. For both α - and β -globin, three recombinants covering all the known chicken globin genes were analysed. In the case of α -globin, the recombinant DNA available comprised the whole gene domain, whereas in the case of β -globin an interruption of 2.1–4.5 kbp (see below) in between $^H\beta$ and $^A\beta$ escaped cloning (J. Dodgson, personal communication).

Figure 1 shows the results of this analysis. The α -gene-type clones, λCaG2 , αG5 and αG6 , all contain A + T-rich linkers. Starting from the 5' end (to the left) of the messenger strand, the map starts with a cluster of three denatured regions of lengths 2,040 (possibly originating from two closely placed linkers), 1,050 and 1,350 bp, separated by 520 and 350 bp respectively of undenatured DNA. The next A + T-rich linker of 1,140 bp is found after the 15.0 kbp of DNA spanning all of the α -globin genes; another A + T-rich linker of 650 bp is located after a further 7.5 kbp of DNA, which includes an unknown region. The cluster of A + T-rich linkers occurs 6.5 kbp upstream from the embryonic π gene; this area includes π' , a further unmapped α -type gene (J. Dodgson, personal communication). In the 3' direction, the single linker bordering the α -globin gene domain is 4.0 kbp downstream from the most 3' $^A\alpha$ gene. Immediately 5' to the π gene we detected another mildly denaturing sequence of ~890 bp; however, in view of its low frequency of denaturation it seems to be qualitatively different from the 800 ± 200 bp A + T-rich linkers defined previously¹.

The gap in the β -gene domain for which no cloned recombinants were available was determined to be 2.1 kbp in the White Leghorn chicken³ and 4.5 kbp in the Brown Leghorn chicken (L.M., unpublished); as we found a value of 2.1 kbp for this region in a further stock of chicken, this area seems to be one of genomic variability. Nevertheless, it is unlikely to contain another β -type globin gene.

Partial denaturation of the DNA of three β -globin recombinants gave the following result. Starting at the 5' end, a single

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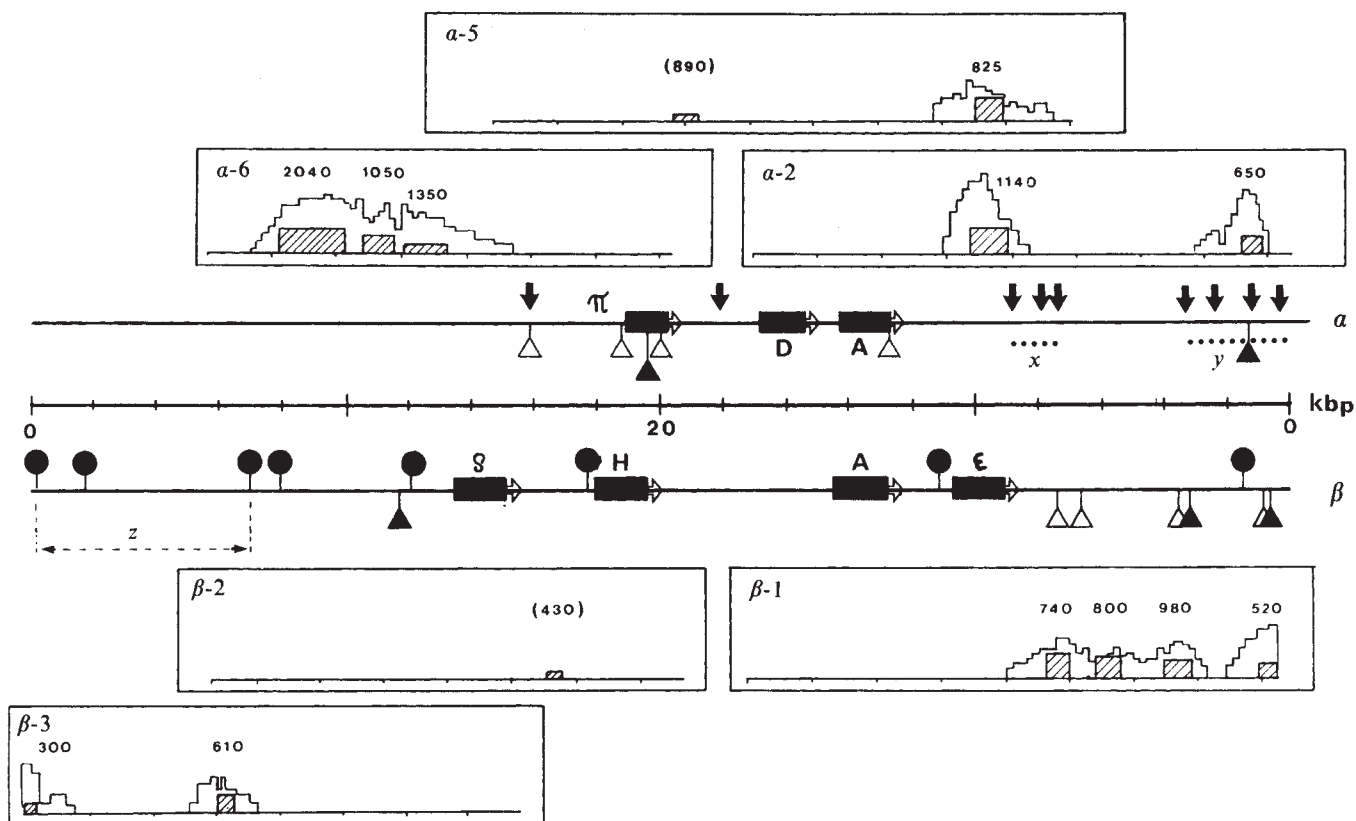


Fig. 1 Globin gene map showing the genomic positions of A + T-rich linkers determined by partial denaturation of cloned recombinant DNA. Insets α -2, α -5, α -6, β -1, β -2 and β -3 show the histograms of frequency of denaturated areas observed plotted against their map position in the cloned DNA, oriented in relation to the long/short arms of the phage DNA. These measurements were realigned against one of the internal denaturated zones; means of frequency of occurrence and size of the A + T-rich zones were calculated and represented in the figure by hatched areas and the bp numbers for any one linker. The genomic maps are taken from refs 4 and 5; from the latter came the positions in the globin domain of an area highly resistant to DNase I (z) and of hypersensitive areas in the α -globin gene domain (x, y). Solid arrows mark the positions of methylated Msp sites; other restriction enzyme sites used to align the various cloned recombinant DNA: Δ , HindIII; $\blacktriangle$, EcoRI; $\bullet$, BamHI. Recombinants from the chick genomic library were λ C α G2, -G5, -G6 and λ C β G1, -G2, -G3 (refs 3, 4).

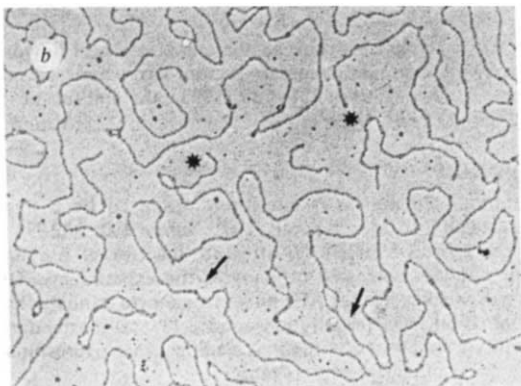
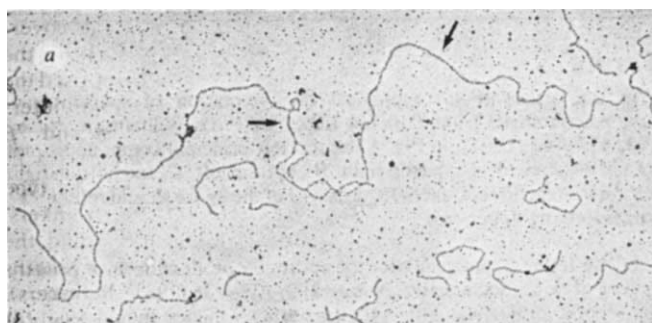


Fig. 2 Partial denaturation of chicken DNA including globin genes cloned in phage λ Charon 4A. DNA was denatured and spread for electron microscopy as described previously¹ and in the text. Arrows indicate the approximate limits of the chicken DNA inserts. *a*, One molecule of clone α -6; the loops include 2,840, 1,290, 1,185 bp; *b*, one molecule of clone α -2; the loops measure 1,590 and 1,210 bp. The small molecules correspond to pBR322 used as length standard.

610-bp linker was observed, which may be part of a cluster placed further upstream, outside the cloned area. With the possible exception of a very rarely denaturing site 430 bp long, there is no other A + T-rich linker in the 14.0-kbp domain covering the embryonic ρ and β genes, which is limited to 5' by the interruption. Beyond this gap there is another 9.0-kbp undenatured stretch covering the adult β and embryonic ϵ -globin genes before a cluster of four consecutive linkers of 740, 800, 980 and 520 bp, separated by 0.94, 1.6 and 2.3 kbp respectively.

In a second approach we analysed a *Drosophila* gene which does not belong to a gene family comparable with that of the globins and which codes for the fairly high-molecular-weight (110,000) ecdysone-inducible P1 protein of the fat body. Its ~ 3.5 -kbp mRNA has been mapped on cloned genomic DNA to the 70 D1-3 region (H. Brock, personal communication) of the 3L polytene chromosome; the gene contains no intervening sequence⁶⁻⁸.

Partial melting of genomic DNA cloned in phage λ Charon 4, carried out as in the experiments reported above, shows that this area is particularly rich in clusters of A + T-rich linkers (not shown); total embryonic *Drosophila* DNA shows melting patterns generally analogous to those of birds and mammals (ref. 1 and J.M., in preparation). The P1 gene maps in the middle of the phage λ Charon 4 recombinant λ Dm 117; the central fragment delimited by HindIII restriction sites was recloned in pBR322 (ref. 8). Figure 3 shows that the P1 mRNA sequence of clone pDmP 1 originates within an A + T-rich linker and runs into a relatively G + C-rich area delimited at a distance of ~ 5.5 kbp by another A + T-rich linker. The pattern shown in Fig. 3 is typical of several other *Drosophila* genes on the same or other chromosomes—LSP-1 β , LSP-1 γ and the P6 genes⁷; in each case the mRNA sequence is in a non-denaturing area framed by A + T-rich linkers (J.M. *et al.*, in preparation).

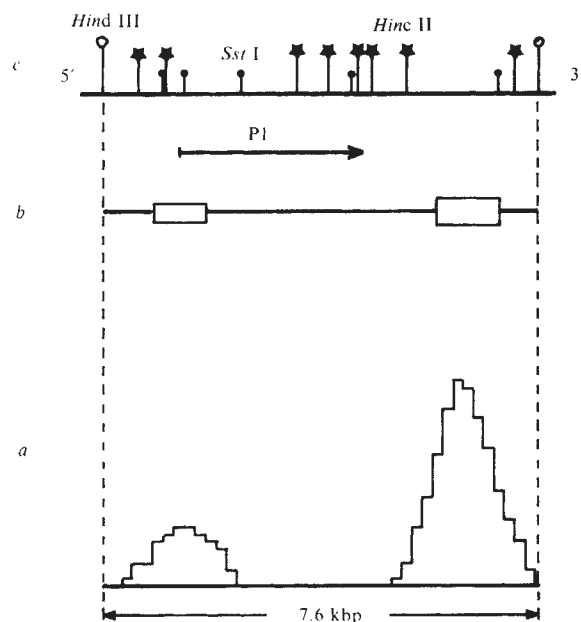


Fig. 3 Partial denaturation of cloned *Drosophila* DNA including the gene for the P1 protein. The DNA of the pBR322 recombinant pDm P1 (ref. 8) was linearized with the *EcoRI* restriction enzyme, denatured as explained in the text in 80% formamide at 24.5 °C, spread for electron microscopy and visualized as explained elsewhere¹. *a*, Histogram of relative frequency of denaturation of any given segment of DNA, in relation to the ends of the molecule including pBR322 DNA. For higher precision the same measurements were realigned with respect to one of the denaturations in the *Drosophila* DNA and recorded as boxes with a length proportional to the average size of the denatured segment and a width proportional to the frequency of denaturation (*b*). *c*, A partial restriction map taken from refs 7, 8 of the cloned fragment used to position the P1 mRNA sequence.

Three main conclusions may be drawn from this analysis. (1) The fundamental features of A+T-rich linkers as observed in total DNA of chicken, duck, rat, mouse, man and *Drosophila* are found in specific cloned DNA fragments as well. In particular, the average size of the A+T-rich zones, and of the domains separating them, falls within the limits defined previously¹. Furthermore, the characteristic homogeneous size of isolated linkers on the one hand and clusters of linkers, including A+T-rich linkers of more variable size on the other was also the case in the cloned DNA. (2) The most striking observation may be the clear-cut framing of both the α - and β -globin gene domains by the A+T-rich linkers. (3) The data on *Drosophila* show that A+T-rich linkers may frame individual genes and thus define putative units of gene function. Furthermore, considering recent sequence data of actin⁹ and histone¹⁰ genes, it may be the general case that genes or gene domains are placed between A+T-rich zones of the type described here.

Two correlations are of interest. First, the ~15 kbp of the chicken α -globin gene domain, as defined by the A+T-rich linkers, correspond to the maximal size of the transcriptional units found at pre-mRNA level for both the α - and β -globin genes in duck¹¹. Recent data^{5,12} have confirmed that in mouse and chick, pre-mRNA exists beyond the CAP-poly(A) boundaries. At the 3' end of the chick α -globin mRNA sequence a further 3 kbp of genomic DNA are co-transcribed⁵; this is about the distance separating, for example, the α gene from the next A+T-rich linker in the 3' direction (compare with Fig. 1). Placing the largest (15 kbp) α -globin pre-mRNA reported¹¹ upstream from this termination point shows that the promoters for these maximal-size transcripts must be in or close to the 5'-framing A+T-rich linker. Second, the mapping of DNase hypersensitive sites, methylation sites and areas of transcriptional activity⁵ also reveals correlations with the A+T-rich linker map given in Fig. 1 (see dotted lines and bold arrows). In the α -gene domain, the 3'-hypersensitive sites almost coincide with the corresponding A+T-rich linkers, and most of the methylated *MspI* sites map within the same A+T-rich linker

areas. A region of high DNase resistance correlates with the area immediately to the left of the A+T-rich linker which borders the 5' end of the β -globin gene domain (dashed line in Fig. 1).

The present data confirm that the A+T-rich linkers are general elements of long-range DNA organization. Furthermore, the clearcut definition of the α - and β -globin gene domains and of the *Drosophila* P1 gene by these linkers suggests that they may have a role as organizational features defining domains of gene function. It is evident that DNA serves multiple functions, not all of which are directly involved in protein synthesis or transcription. The A+T-rich linkers, occurring outside the coding and transcribed areas, might also be involved in DNA functional organization at the chromosome/genome level.

We thank Drs J. Dodgson and D. Engel for their library of cloned chicken DNA and the globin gene-containing clones, Helene Grimal for technical assistance, and members of our team and of the staff of the EM laboratory at the IRBM (director Dr L. Benedetti). We particularly thank Mrs Chantal Cuisinier and R. Schwartzmann for help in preparation of this manuscript. This work was supported by the French CNRS, Délégation Générale à la Recherche Scientifique et Technique, Fondation pour la Recherche Médicale Française and Institut National de la Santé et de la Recherche Médicale.

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Errata

In the article 'Palaeontological documentation of speciation in Cenozoic molluscs from Turkana Basin' by P. G. Williamson, *Nature* **293**, 437–443 (1981), in Fig. 3 the top left principal components plot should be labelled 'A', the top right 'B', bottom left 'C' and bottom right 'D'. In the legend references to parts a–d of the figure should refer to the corresponding upper case.

In the letter 'Autoregulation of androgen production in a primary culture of rat testicular cells', *Nature* **293**, 737–738 (1981), the name of the second author was misspelt; the authors' names should read 'Eli Y. Adashi & Aaron J. W. Hsueh'.

In the article 'Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumour virus chimaeric plasmids' by F. Lee *et al.*, *Nature* **294**, 228–232 (1981), the first sub-heading on page 232 should read 'Mapping the 5' end(s) of MMTV-*dhfr* RNA'.

Corrigenda

In the letter 'Immune (γ) interferon produced by a human T-lymphoblast cell line' by I. Nathan *et al.*, *Nature* **292**, 842–84 (1981), some of the reported interferon titres are inaccurate. A commercial interferon standard was used in many of the experiments. This preparation was checked against NIH human leukocyte standard; however, some of the commercial preparations used as laboratory standards were subsequently found to be defective, leading to an overestimation of interferon titres of approximately tenfold.

In the letter 'Expression of a human gene for interferon in yeast' by R. A. Hitzman *et al.*, *Nature* **293**, 717–722 (1981), refs 10 and 18 should read:

- Faye, G., Leung, D. W., Tatchell, K., Hall, B. D. & Smith, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2258–2262 (1981).
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MATTERS ARISING

Guidance system for pheromone orientation in moths

THE explanation proposed by Kennedy and co-workers¹ of a new guidance system for pheromone orientation contains several conclusions inconsistent with previous publications: (1) the conclusion that "persistent upwind flight requires decreases as well as increases in the pheromone stimulus" conflicts with the behaviour reported for another moth *Anagasta kuhniella*², and the persistent upwind orientation of flying *Drosophila*³, walking silk moths⁴, cockroaches⁵ and grasshoppers⁶ to uniform and constant odours. Possibly the sustained upwind flight could not be elicited in *Adoxophyes orana* because the concentration in the cloud was too low: "at least 30 times lower than the average concentration in the plume".

(2) The hypothesis that zig-zagging and casting flight is a programmed function of pheromone concentration and that "the amplitude of these cross-wind movements change(s) inversely with the strength of the pheromone stimulus" cannot be supported critically by only the anecdotal comparison of *A. orana* flight without pheromone against that in a single pheromone concentration. Alternatively these observations might only be associated with the presence or absence of pheromone.

(3) That "zig-zagging and casting flight is not a response to loss of pheromone" is not adequately supported by noting "frequent track reversals inside the pheromone corridor". An alternative conclusion is that not all turns are elicited by the same mechanism. Reversal turns not related to a plume boundary have previously been reported in other insects^{4,5}, but this does not imply that turns made specifically at the boundary or after a change in the pheromone concentration are not elicited as a stimulus response. For example, cockroaches turn both at the plume boundary and at irregular points within the plume⁷.

An integrated orientation system rather than the models discussed by Kennedy and co-workers¹ would be more consistent with present information. In an integrated system, several factors contribute to the control of the rate and patterns of turning, including compensatory turns made to correct deviations from the preferred wind angle, reversal turns made at the plume boundaries, stochastic turns, and turns initiated internally at a regulated frequency if a turn has not yet been elicited by one of the previous factors. In addition, the concentration of pheromone must also regulate the flight speed and the preferred wind angle.

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KENNEDY ET AL. REPLY—The new guidance system we proposed is admittedly inconsistent with earlier publications, not least our own, and was therefore not lightly arrived at. Tobin and Bell's numbered points are discussed briefly below but reference should also be made to the full account of this work which appears elsewhere¹.

(1) In the long run some inconsistencies will surely turn out to reflect real differences between cases. However, for the moment note that the only two published examples^{2,3} of persistent upwind flying—as distinct from walking—in uniform odour are anecdotal. Our reason for mentioning the non-persistent upwind flights in the very low concentration of our tunnel-filling pheromone cloud, was existing evidence from other moths^{4,5} that upwind flight is retarded by high rather than low pheromone plume concentrations. However, we also mentioned non-persistence in our side 'corridor' pheromone cloud, and the concentration in that was higher than the average in the broken pheromone plume where upwind flight was persistent¹. Thus we concluded that the non-persistence recorded was due to the continuity of stimulation in the unbroken pheromone clouds, not the concentration.

(2) The quoted remark did refer only to the presence or absence of pheromone, but it rested on the non-anecdotal¹ finding that onset of the pheromone stimulus reduced the amplitude of cross-wind movements while cessation of the stimulus increased it; and, in another flying moth, changing the concentration of pheromone present has likewise been shown to produce an opposite change in amplitude⁴.

(3) We expected, but did not find, some evidence of pheromone loss inducing cross-wind reversal of the flight track by *Adoxophyes orana*. Of course, we cannot say it never happens, but we can say that the probability of such a reversal increases significantly on entering pheromone¹; and in another moth cross-wind movements had already been shown to lengthen on leaving pheromone⁵.

Like Tobin and Bell, we regard this

guidance system as a highly integrated one^{5,6} but go further in regarding the stochastic reversal turns, not as a separate class of turn, but as turns initiated internally at a frequency that is regulated stochastically by the pheromone inputs.

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Tilt of the central gas disk of the Galaxy

BLITZ ET AL.¹ have suggested that the tilt of the central galactic gas disk might be explained by a bending wave instability due to a gravitational interaction between the thin gas disk and the central stellar bulge. However, the instability theory due to Bertin and Mark² on which this suggestion is based, is inapplicable in the case of the central gas disk. For the theory to apply, the vertical gravitational forces on the gas disk must be due largely to its own self gravity with only a small contribution from the bulge. The frequency of the bending waves of a thin self-gravitating disk is then given by^{2,3} $(2\pi G \Sigma k)^{1/2}$ where Σ is the surface mass density and k is the radial wavenumber of the bending wave, yielding the group velocity used by Blitz et al.¹ to predict the scaling law for the height of the bend (h) as a function of radial distance from the galactic centre (r).

However, the gravitational force experienced by the gas disk between $r = 300$ pc and 2 kpc is dominated by the stellar potential. The central stellar bulge is in fact significantly flattened⁴ (major and minor semi-axes are 2.5 and 1.3 kpc), while the stellar disk component (thickness ~ 500 kpc) is not completely negligible in the central region⁴. The magnitude of the vertical gravitational acceleration at height h due to the stellar potential is therefore approximately that of an oblate spheroid with mass density ρ_* and eccentricity close to unity⁵, that is, $4\pi G \rho_* h$. Whilst the magnitude of the vertical acceleration due to the self gravity of the bent disk can be estimated² to be $2\pi G k \Sigma h$. Using $\rho_* = 6 M_\odot \text{pc}^{-3}$ (ref. 6) and $\Sigma = 6 M_\odot \text{pc}^{-2}$ (ref. 7) at $r = 1$ kpc, the ratio of the stellar field acceleration to that of the gas self gravity is λ/π , where λ is the wavelength of the bending wave in parsecs. Because $\lambda \sim r$ for the tilt, the

stellar force dominates by a factor $\sim 10^2$ – 10^3 , and hence the self gravity of the disk is negligible. Therefore it is invalid to use the Bertin and Mark theory.

A more appropriate approximation would be to consider the bending modes of a gas disk due to motion in the stellar potential alone. The modes obtained from such an analysis⁸ which are most closely analogous to the bending wave of Bertin and Mark have a frequency of $\sim (4\pi G\rho_*)^{1/2}$ yielding a very small group velocity in comparison with the phase velocity.

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BLITZ AND MARK REPLY—Nelson's suggestion that the Bertin and Mark¹ bending wave theory is inapplicable to the dynamics of the gaseous disk at the galactic centre seems to be based on an incomplete analysis of the singular perturbation problem involved when a disk of small (but not altogether negligible) mass is immersed in a larger spheroidal component.

For disk mass much smaller than spheroidal masses, it is not appropriate to omit the disk mass terms. The 'kinematic waves' resulting from this omission (see equation (D7) of (ref. 1) dominated by ν_z^2 on the right-hand side) dissipates on the rather short differential rotation shearing time scale $|\mathrm{d}\Omega/\mathrm{d}\ln r|^{-1}$. On the other hand, including the dynamical effects of the small disk mass results in self-gravitating waves with the much longer group propagation dissipation time, $\sim 2R\mathrm{d}k/\mathrm{d}\omega$, which has the dispersion relation (D7) of (ref. 1). The neglect of similar terms in Nelson's² formalism for modes symmetric about the galactic plane would fail to produce the familiar Jeans instability (the bending waves are the antisymmetric disturbances about the galactic plane). By contrast, Bertin and Mark have included terms of the 'sound wave' type included by Nelson, but within the context of galaxy warps, stellar dispersive speeds squared are much larger than the corresponding terms in the gas.

In any case, Bertin and Mark have included terms which reflect more completely the dynamics of the disk. The conclusion with or without these additional terms is that the scaling law ($r\Sigma^2h \sim \text{constant}$) used by us is consistent with the observations of the inner disk of the

Galaxy as well as the warps of the outer region of several galaxies as shown by Lake and Mark³.

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Lipoprotein assembly in *Xenopus* yolk-platelet crystals

FROM his determination of the space group of yolk-platelet crystals from several new species, Lange¹ has questioned earlier data of ours for similar lipoprotein crystals from *Xenopus*². The new data are based entirely on electron micrographs from fixed sectioned specimens, a procedure we intentionally avoided during our studies. This evidence as well as a few other statements made in the paper need to be scrutinized.

The first data shown (Fig. 1 of ref. 1) are three 'tracings' of electron diffraction patterns from yolk crystals of the species *Triturus*. It is unfortunate that Lange did not publish the patterns themselves because frequently the high noise level from such poorly diffracting specimens makes interpretation of the patterns difficult. Furthermore, a more analytical description including the averages and standard deviations of the observed lattice spacings would have been useful. It is, after all, the unit cell spacings which we and now Lange, have used to identify specific projections^{1,3}.

In further criticism of the data presentation, the space group determination using Figs 2 and 3 of ref. 1 is confusing because no optical diffraction patterns are shown and the micrographs were obtained from two different species. Lange's Fig. 3, for example, bears a strong resemblance to the *hol* zone from lipoprotein crystals of *Xenopus*³. Lange describes this as the *hko* zone, suggesting that simple relabelling of the *b* and *c* axes would make the results consistent with the earlier work³. Indeed, we agree that the projection shown in Fig. 3 should have *pgg* symmetry. As the *b* and *c* axes in the unit cell for *Xenopus* are roughly the same length, they may equally well be similar in other species and could easily be interchanged. Unit cell dimensions can be precisely obtained for wet and dry crystals by using X-ray powder methods³.

In image reconstruction studies, the best way to determine the symmetry of a given projection is to examine the phases of strong symmetry-related reflections⁴. In the studies of the lipoprotein complex from *Xenopus*, phases of each centric projection were calculated and were then shifted to minimize the difference between that calculated and the nearest real value^{2,5}. Two pairs of strong

reflections on the 001 projection (1, 3, 0 and $1, \bar{3}, 0$; 2, 3, 0 and $2, \bar{3}, 0$) had phase relationships consistent with only *pmg* symmetry⁵. Six pairs of reflections had phase relationships consistent with *pgg* symmetry for the view we assigned to the *hol* projection. Many of the projections used in the image reconstruction were studied earlier by optical filtering methods. Even on such filtered images, both the number of molecules and the symmetry can be difficult to ascertain⁶.

Furthermore we take issue with Lange's discussion of the reliability of positive versus negative staining¹ in special reference to our image reconstruction results. Certainly the appearance of lattice micrographs will be different when one is positively stained and the other negatively stained. However, the immobilization of stain (positive staining effects) adjacent to solvent channels filled with stain (negative staining), is unlikely to produce major artefactual results. Thus, we emphasize the importance of negative stain as well as the lack of chemical fixation in the earlier study².

Finally, we note that although the work on *Xenopus* was severely limited by the chance finding of different crystal fragments, it still contained data from 10 different views selected from several hundred electron micrographs^{2,3}.

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LANGE REPLIES—If the symmetric lipoprotein aggregates in yolk platelets of higher anamniotes are orthorhombic crystals as reported for *Xenopus laevis*¹, their space group is $P2_12_1$, as documented by symmetry *pgg* of their axial projections² at usual electron microscope resolution in eight different species above the cyclostome level (among them *X. laevis*).

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BOOK REVIEWS

Newton's achievements: from rumour to fact

Richard S. Westfall

VOLUME VIII, which, except for a promised volume of indexes, concludes D.T. Whiteside's monumental edition of *The Mathematical Papers of Isaac Newton*, is more of biographical than of mathematical interest. It takes up the final desultory efforts of a man who had recently celebrated his fifty-fourth birthday when the volume commences and had also chosen to fill his life with the administrative minutiae of the Royal Mint instead of the labours of a Lucasian Professor of Mathematics. The volume contains very little original mathematics; almost all of the mathematical activity it displays was provoked by the immediate demands of the battle with Leibniz over priority in the invention of the calculus. Fittingly, it concludes with a draft of an intended preface for the 1722 edition of *Commercium epistolicum*, the volume of letters and papers that Newton himself had secretly compiled and edited and that the Royal Society had published in 1712 as the report of an "impartial" committee appointed to investigate Leibniz's complaint. When he wrote that preface, Newton was approaching 80 years of age. As he himself once said, no old men (excepting Dr Wallis) love mathematics, and it is not surprising that Whiteside finds nothing to include from his remaining five years of life.

Do not mistake the paragraph above as criticism of Vol. VIII. A complete edition of Newton's mathematical papers could not neglect these manuscripts, and to say that they are more of biographical than of mathematical interest is in no way to suggest that they have no interest at all. It is part of the editor's achievement that he infuses into their publication learning equal to that which he devoted to Newton's earlier creative endeavours in mathematics. Thus he conducts us through Newton's responses to the two challenge problems posed by Bernoulli, the initial publication during the first decade of the eighteenth century of various papers written earlier, relevant aspects of the second edition of the *Principia*, and the venomous exchange with Leibniz and his followers on the issue of priority which spread out over a full decade. Whiteside's presentation of the correction of Proposition X, Book II, for the *Principia*'s second edition is a *tour de force* in the full sense of the phrase. From Newton's papers he has assembled the sheets on which Newton confirmed the validity of Bernoulli's objection, and he shows in detail the exact nature of the error in

The Mathematical Papers of Isaac Newton Vol. VIII, 1697–1722. Edited by D.T. Whiteside. Pp. 704. ISBN 0-521-20103-9. (Cambridge University Press: 1981.) £85, \$195.

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Newton in old age — a portrait painted not long before his death in 1727.

edition one, which Bernoulli himself had not understood correctly. Whiteside then proceeds through six successive attacks on the problem by Newton (plus seven other draft passages). He pinpoints Newton's final location of his error and its correction, together with an alternative demonstration of the correct result, and he argues persuasively that only an unperceived computational error prevented Newton from realizing that he could have amended the proposition within the framework of the original demonstration without the complete redrafting that he finally inserted (to his embarrassment) in edition two.

Whiteside concludes the volume and the edition with 160 pages of documents from Newton's own extended effort to defend his right as true inventor of the calculus. Not long ago, in Vols VI and VII of Newton's *Correspondence*, an extensive documentary history of the priority dispute was published. Naturally the focus was on correspondence, including many hitherto unpublished letters from the Continent. The drafts of Newton's defence of his priority that Whiteside publishes thus supplement, rather than repeat, the *Correspondence*. His lengthy intro-

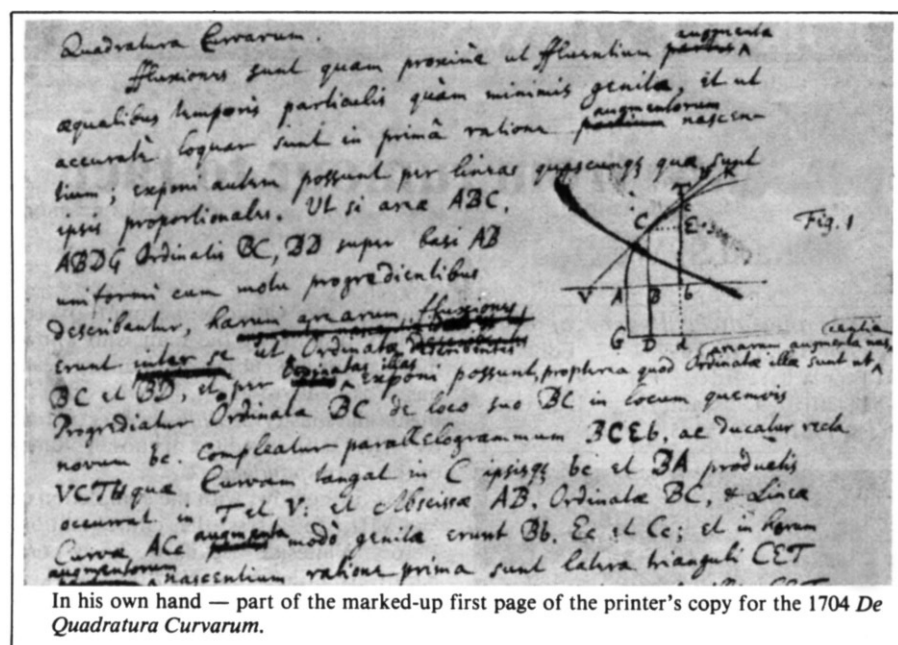
duction, the valuable account of the priority dispute by the man who knows more about the subject than anyone ever has or is likely to again, likewise complements the masterful *Philosophers at War* by A.R. Hall, the editor of those volumes of the *Correspondence*.

It is fitting now, with the completion of Vol. VIII, to assess the entire edition. Before Whiteside began, we knew comparatively little about Newton's work in mathematics. As Whiteside states in the preface to the present volume, Newton failed to impress the fact, as distinct from the hearsay, of his mathematical achievement on his contemporaries. The period of more than two centuries that followed Newton's death did not amend that state of affairs. Furthermore, Newton did not arrange his papers to facilitate an editor's work. Only Newton scholars who have worked on the manuscripts themselves can appreciate the initial stage of Whiteside's labour, finding his way through what he aptly describes as "the disordered limbo of Newton's private papers".

Volume VIII offers two striking displays of the extent to which he has mastered these difficult manuscripts, though one could find similar examples in every volume. He has identified an unlabelled page of miscellaneous calculations as Newton's demonstration of the essence of Fatio de Duillier's novel solution of Bernoulli's problem on the line of quickest descent (pp. 86–91). And from sheets dispersed in both the Portsmouth Papers in the Cambridge University Library and the Macclesfield Papers in Shirburn Castle he has reassembled much of the manuscript for a treatise on fluxional analysis that Newton composed to append to the *Principia*'s second edition. The papers were separated soon after he dropped the idea; thus Whiteside has resurrected an essay which has not existed as a single entity since its composition nearly 270 years ago (pp. 258–296).

Impressive as his achievement in arranging the manuscripts is, it pales in comparison to the real work of infusing them with understanding so that the invention of the calculus and a great deal more once again spring to life. In effect Whiteside has done for Newton what Newton failed to do for himself; he has transposed his mathematical achievement from the realm of rumour to the realm of fact. Because of the inherent difficulty of the material, there is no way in which such a work can be made easy for the reader, and

National Portrait Gallery



because Whiteside has brought a high level of mathematical expertise to his task, the edition is doubly difficult. I myself have criticized earlier volumes on this score, arguing that the technical level of the edition effectively closes it to all but a tiny handful of readers. I have come to the conclusion that this criticism was ill-considered, and I take this occasion to apologize to Whiteside. Nonetheless, I still hold the idea that animated it: that it will be a tragedy if the knowledge the edition makes available of Newton's achievement in mathematics, one of the supreme triumphs of the human spirit, does not reach beyond the confined circle of professional historians of mathematics. As it now appears to me, Whiteside has done quite right in treating the papers with a level of sophistication that matches their own. It belongs to others, including Newton scholars such as myself, to translate the achievement into a broader idiom without vulgarizing it. If we fail in the task, we, rather than Whiteside, should be the object of criticism. With this magnificent edition, we cannot complain of an inadequate source.

In a work of such magnitude, there are bound to be — I will not say errors — but a few things that seem to me to be misjudgements. In Vol. VII, annoyed by Newton's apparent revulsion from modern analysis in the 1690s and by his preference for classical geometry, Whiteside has put together a work on *Geometria* in two books, which seems to show that Newton reversed himself once more to embrace analysis anew at the end of his active career in mathematics. By comparing the original manuscripts, I have convinced myself that in this case Whiteside has created an artificial work that never existed, joining a manuscript from the 1680s to a totally separate one from the 1690s in order to portray Newton as he would prefer him to have been. It seems to me, further, that he

made a serious mistake in not including the two *epistolae* written in 1676 for Leibniz, even if their texts (though not Whiteside's commentary) are readily available in the *Correspondence*. In an edition notable for its completeness, their absence is the only major omission. Third, I think he erred in substituting the form $\dot{x}$, $\dot{y}$ and $\dot{z}$ for Newton's m , n and r in his translation of *De methodis* of 1671. "Pricked letters", as Newton called the distinctive notation he later devised, assumed some significance in the priority dispute. Readers tend always to use an available translation, and they are apt to be misled by it in the present case. I could list a few more possible defects; but if I have reached this level of triviality with my third, it should be obvious how few and how small I take them to be.

A few years ago I hailed Whiteside's work as the finest edition of scientific papers ever published. I now think that judgement was inaccurate. It erred in being too restrained. I am convinced that *The Mathematical Papers of Isaac Newton* is one of the outstanding scholarly achievements of the twentieth century. Where other editorial projects concerned with less difficult materials have created veritable factories, Whiteside has equipped himself primarily with a writing pad, a pencil and, to restrict himself more fully to his study, a stack of xeroxes. Upon the latter he has exercised an extraordinary level of technical expertise joined to an extraordinary level of devotion. How seldom do the two qualities, raised to such a degree, come together. With them, Whiteside has built what every true scholar dreams of constructing, a monument which will endure through the centuries as part of mankind's precious legacy of learning. □

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Iron in the soul

Walter Gratzer

The Nobel Duel. By Nicholas Wade. Pp.321. ISBN 0-385-14981-6. (Anchor Press/Doubleday, New York: 1981.) \$15.95.

ONE of the elders of modern biochemistry once recounted how, in the days before peer-review was invented, his benefactor would from time to time visit the laboratory to see what use was being made of his largesse. He inquired on one such occasion whether he could be of any further help and, being told that ten dollars for the purchase of frogs would be welcome, he drew out his wallet, laid two five-dollar notes on the laboratory bench and took his leave.

Well, the past, it has been said, is another country, where they order their affairs differently. The pursuit of the pituitary releasing factors, which is the subject of Nicholas Wade's book, consumed many millions of dollars, the undivided efforts of postdoctoral research fellows, students and technicians in something approaching battalion strength and, according to one estimate, the brains of five million sheep in the isolation of thyrotropin releasing factor alone. The investment was all but wasted: in 1969 the Study Section of the NIH that had supported the two duellists of this story, Guillemin and Schally, through many years of seemingly unproductive toil, came close to deciding that enough was enough. In the words of a member of the committee, the programme was "within an eyelash of being abandoned". The releasing factors had been compared by a respected endocrinologist to the Loch Ness Monster, and Guillemin and Schally had brought ridicule on themselves by an obsessive antagonism that regularly expressed itself in public brawls at conferences.

Sustained by the manic intensity that distinguishes the barons of science from the cannon-fodder in their campaigns, both survived. Each, on the testimony of this account, has an ego like a raging ulcer, as much sensitivity or remorse as a runaway locomotive and awesome reserves of single-mindedness, tenacity and courage. They have in superabundance what Noel Coward defined as the secret of success — the capacity to survive failure. But, in the words of a discarded collaborator of Schally's, such men "are not lovable types". Whether on balance they serve science well, the reader will have to decide for himself.

Nicholas Wade has an engrossing tale to tell and does it in masterly fashion. He does not allow the interplay of personalities that provides much of the drama to obscure the science, which he handles with enviable assurance. No less remarkable is the way in which he has made his cast of characters speak for themselves and has drawn from

their mouths reflections of marvellous candour.

In the beginning was Geoffrey Harris, the English physiologist, who first postulated that the brain directs the release of pituitary hormones by despatching chemical messages from the hypothalamus to the pituitary. Guillemin and the Canadian endocrinologist, Murray Saffran, with whom Schally was then working as a technician, obtained early and independent evidence of the existence of such factors, and there ensued an uneasy collaboration between Schally and Guillemin that soon gave place to an ungovernable rivalry. According to *The Devil's Dictionary* calamities are of two kinds; misfortune to ourselves and good fortune to others. Guillemin and Schally dogged each others' footsteps through success and failure, each cast down by the other's victories and elated at his defeats; when, for example, Schally's group took for a releasing factor a proteolytic fragment of myoglobin, which they laboriously isolated and sequenced after processing a quarter of a million brains, Guillemin did not conceal his pleasure.

In the laboratory it seems that both Guillemin and Schally (though both had proved their ability as young men) made only minor contributions to the work of their groups, but it was they who kept their groups in being. The burden of the science was borne by a superlatively capable corps of young men — Burgus and Vale, Ward and Brazeau for Guillemin; the Japanese, Arimura, Matsuo and Baba, together with Radding, Carter and Nair for Schally. Their efforts were ill-rewarded — indeed, on the evidence here, it is small wonder that a postdoctoral liberation movement of a kind appears to be emerging in the United States.

Both teams had remarkable achievements. One that stands out is a dazzling feat of peptide chemistry by the Japanese visitor, Matsuo, who determined the sequence of LRF by simultaneous analysis of unfractionated, overlapping proteolytic fragments on 50 µg of material, and so won the race for Schally.

The Nobel Duel is a hugely entertaining read, but it is also much more, for it lays bare the mechanics of Big Science (into which biology is being increasingly assimilated), and exposes its virtues and blemishes to public view. Whether the process of discovery feeds on such ferocious competitiveness as is here on display is an interesting and indeed important question. Wade opines that other highly regarded workers in the field, such as Saffran and Hearn, had too much of a

sense of humour and of the fitness of things to engage in such destructive proceedings; this fastidiousness, just as a classical education has been alleged to do, enabled them perhaps to despise the success that it prevented them from achieving. Nonetheless, it is remarkable how close other laboratories with vastly smaller commitments and funds, such as that of Harris, came to a solution of the problem.

A piquant footnote to the history of the long haul to the structure and function of

the releasing factors is the reaction of an early adversary of Harris's, that most entrenched of all the illuminati of the scientific establishment, Lord (Solly) Zuckerman. He, it seems, remains adamant that Harris was wrong from the outset and that the pituitary hormone releasing factors are, after all, no more than a mirage. □

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Mysteries of the zeta potential examined

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Zeta Potential in Colloid Science: Theory and Application. By Robert J. Hunter. Pp.386. ISBN 0-12-361960-2. (Academic Press: 1981.) £35, \$84.

ELECTROKINETIC phenomena, such as streaming potential and electrophoresis, have been known for over a century. They arise whenever there is relative motion between a charged interface and a liquid and are usually interpreted in terms of the zeta potential, which is conventionally defined as the electric potential at the plane of shear. There has always been an air of mystery surrounding the concept of zeta potential, partly because of theoretical problems concerning the structure of the electrical double layer and the precise location of the shear plane within it, and partly because of difficulties in obtaining reliable experimental values. Although there have been several books on electrokinetic phenomena generally, Professor Hunter's is the only one, to my knowledge, which is devoted to the enigmatic zeta potential itself.

To a large extent, the book is successful in drawing together and rationalizing a vast amount of material covering many aspects of the subject. It should be essential reading for those embarking on research in the area and most established practitioners will want to have a copy within reach, or at least in the library of their institution.

After a brief, scene-setting introduction, the author first deals with the distribution of charge and potential at interfaces. Most of this material is readily available elsewhere and the treatment here offers no new insights. However, since it forms the basis of much of what follows, it could not reasonably have been left out.

By far the most useful chapters are those concerned with experimental techniques and the calculation of zeta potentials. There is an exceptionally clear and up-to-date exposition of the procedures for obtaining zeta potentials from electrophoretic mobilities. The experimental chapter includes a wealth of practical detail, such as the design of electrophoresis cells, and even advice on how to avoid

leakage problems with platinum streaming potential electrodes.

The chapter on electroviscous and viscoelectric effects is an admirable survey, a task which does not seem to have been previously attempted. Hunter's conclusion from the discussion of viscoelectric effects is that the electrokinetic plane of shear cannot lie too far from the outer Helmholtz plane and that the zeta potential must be nearly equal to the potential just inside the diffuse layer. This will comfort the many colloid scientists who have been making similar assumptions for years; but those who have advocated comparatively thick layers of structured water at interfaces may still remain unconvinced.

The remainder of the book is concerned with applications of zeta potential and the effect of simple inorganic ions and more complex adsorbates. The discussion of oxide surfaces raises the important question of the distinction between points of zero charge and isoelectric points, but, unfortunately, in an uncharacteristic lapse from his usually clear style, the author creates some confusion, especially with regard to the role of specifically adsorbed ions. There are mentions of applications in such areas as colloid stability, flotation and electrophoretic deposition, but they are too brief to do justice to these various subjects.

It becomes evident from the final chapters that there are still major uncertainties concerning the nature of the electrical double layer at solid-liquid interfaces. In some cases, experimental values of charge density and zeta potential can only be reconciled with current models by making rather unrealistic assumptions, as the author readily admits. Perhaps it is time for a thorough reappraisal of concepts such as the Stern layer and molecular capacitors, bearing in mind that on this scale (c. 1 nm) water is not a structureless medium and that real surfaces are almost never smooth. □

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The second volume of Colin Ronan's *The Shorter Science & Civilisation in China* has just been published by Cambridge University Press. The book is an abridgement of the material in Vols III and IV/1 (for reviews see *Nature* 186, 36 and 196, 844) of Joseph Needham's original text, and costs £15.

Single-system geology after Arkell

Janet V. Watson

Lower Palaeozoic of the Middle East, Eastern and Southern Africa, and Antarctica: With Essays on Lower Palaeozoic Trace Fossils of Africa and Lower Palaeozoic Palaeoclimatology. Edited by C.H. Holland. Pp.331. ISBN 0-471-27945-5. (Wiley: 1981.) £38, \$84.

"IT HAS been my personal experience that anyone seeking to understand a single geological system must flounder in the literature for some twenty years. . . . Users of this book will be saved some twenty years work". This wry statement comes from the preface to W.J. Arkell's *Jurassic Rocks of the World* (Oliver & Boyd, 1956), the first general synthesis of a single system on a world-wide basis. During the quarter-century since its publication the need for regional or global syntheses has increased, and the volume under review is therefore sure of a welcome. It is the third of an ambitious series focused principally on the Cambrian, but taking in Ordovician and Silurian rocks where the nature of the evidence makes precise chronostratigraphy difficult. The regions covered in this volume — the Middle East, north-eastern and southern Africa and Antarctica — are as difficult as any and the decision to cover all of the Lower Palaeozoic systems seems wise.

In introducing his now classic volume on the Jurassic, Arkell outlined two objectives:

. . . to present a unified description as a reference work and . . . to enquire what can be deduced as to faunal realms, climate, palaeogeography, volcanic and plutonic activity and earth movements . . . during one reasonably circumscribed period of geological activity.

Accepting these as the true objectives of any comparable work, one must say immediately that the principal authors here (R. Wolfart, E. Klitzsch, I.C. Rust and M.G. Laird) have dealt nobly with the reference aspect. They have brought together details of successions in some of the least-visited and least hospitable terrains on Earth and have provided valuable correlation tables and palaeogeographic maps. Their chapters will remain the standard references for the Middle East, Libya and adjacent territories, southern Africa and the Ross geosyncline for years to come. As a bonus, there are interesting chapters on Lower Palaeozoic trace-fossils in Africa (T.P. Crimes) and palaeoclimatology (N. Spjeldnaes).

But what about Arkell's second objective? The unity of outlook and stylistic grace which he brought to the Jurassic are perhaps unlikely to be achieved in a volume with many authors: one could, however, justly look for a broad treatment of geological events in Lower Palaeozoic times. A four-page introduction by R.M.

Shackleton reminds us that the areas dealt with are linked by and partially coincide with one of the world's major systems of mobile belts — the Pan-African — which was active immediately before and sometimes during the Cambrian period. Scarcely a mention of this relationship is to be found in the rest of the book, with the honourable exception of the Antarctic chapter. An opportunity seems to have been lost here, though the long-suffering editor (C.H. Holland) whose frustrations are outlined in the preface, may well protest that to have asked for a broader treatment would have made his labours impossible.

Nonetheless, a fuller introduction or a final review chapter might have made all the difference. Stratigraphy is the senior branch of historical geology, but can it any longer afford to close the door on tectonics, metamorphism and magmatism? □

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Landmark of early man in Britain

Paul Mellars

The Lower and Middle Palaeolithic Periods in Britain. By Derek A. Roe. Pp.340. ISBN 0-7100-0600-4. (Routledge & Kegan Paul: 1981.) £35, \$95.

NO ONE is better qualified to write a book on the earliest stages of human occupation in Britain than Derek Roe. For the past 20 years he has been involved in detailed first-hand work on the sites of this period, and has produced not only the authoritative gazetteer of the major Palaeolithic localities in Britain, but also the most exhaustive typological analysis of the archaeological assemblages themselves. The appearance of this general survey of the earlier Palaeolithic material must therefore be regarded as a major landmark in studies of the period in Britain.

Two features of the book are particularly impressive: its evident authority in dealing with the difficult interpretative issues involved and its readability. Dr Roe writes with a degree of clarity, style and fluency which is all too rare in archaeological literature. Some might say that the book is over-written in places, but this kind of judgement must depend to a large extent on the kind of readership which is anticipated. Dr Roe has clearly aimed at an

audience which includes not only the specialist prehistorian but also the "interested layman", and for this mixed readership his style of presentation is probably ideal.

To pick out particular features of interest in a book of this scope is an almost impossible task. Perhaps the most significant single feature which emerges from the discussion is the rapidly increasing evidence for the occupation of southern Britain by hand-axe-using communities at some point substantially prior to the Hoxnian interglacial. Roe's arguments for believing that sites such as Fordwich (Kent) and Kent's Cavern (Devon) belong to this early phase are to my mind almost conclusive, and for these sites an age of at least 350–400,000 years would, from several lines of evidence, seem reasonable. Equally interesting from a somewhat different standpoint is the rather curious dichotomy between hand-axe and non-hand-axe ("Clactonian") industries which has been documented for the Hoxnian interglacial itself. This dichotomy — probably clearer in Britain than anywhere else in Europe — remains one of the most intriguing problems in Palaeolithic archaeology, for which a really convincing explanation still remains to be found.

Evidence for the later stages of Lower and Middle Palaeolithic occupation is still infuriatingly vague. Uncertainties of stratigraphic correlation, coupled with the lack of any reliable technique of absolute dating, renders any analysis of occupation during the Wolstonian and Ipswichian periods exceptionally difficult. As Roe points out, there is a wide variety of industrial variants which must belong at some point within this time range, but in the absence of reliable dating any discussion of the meaning of this variation (chronological? cultural? functional?) must remain largely hypothetical and the question of interpretation open.

One criticism that might perhaps be levelled at the book is that at some points it verges on the parochial, paying only limited attention to contemporaneous developments in other parts of Europe. In the assessment of the earliest Palaeolithic industries this kind of comparison would certainly have been useful, and perhaps even more so in the discussion of the Middle Palaeolithic sites. But this is at most a minor criticism and can hardly detract from the overall value of the book. Dr Roe's major achievement is to have brought together a remarkably wide-ranging and diverse body of data, and to have presented it in a clear, well-integrated and easily accessible form. By performing this task, he has produced what is clearly a classic of British prehistory that will serve as the definitive account of this period for many years to come. □

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